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**TECHNICAL REPORT NO.
DATE**

**IMPACT OF SLEEP RESTRICTION ON LOCAL IMMUNE RESPONSE, SKIN
BARRIER RESTORATION, COGNITION, MARKSMANSHIP AND GUT FUNCTION
WITH AND WITHOUT 'MULTI-NUTRIENT' NUTRITION INTERVENTION.**

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**United States Army
Medical Research & Materiel Command**

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Impact of sleep restriction on local immune response, skin barrier restoration, cognition, marksmanship and gut function with and without ‘multi-nutrient’ nutrition intervention

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Running Title: Immune response to sleep restriction and nutrition

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Authors’ contributions

Dr T. Smith conceived of and designed the study, collected the data, conducted the analyses and drafted the manuscript. Mr. Wilson collected the data and assisted in the data analyses related to immune function and skin barrier restoration. Dr. Karl contributed to study design and revised the article for important intellectual content. Drs. Heaton, C Smith and Cooper assisted in study design and contributed to data collection. Dr. Orr was responsible for data collection and analyses related to flow cytometry. Ms. Haskell and O’Connor were responsible for data collection related to dietary intake. Dr. Hruby conducted statistical analyses to identify predictors of skin barrier recovery. Drs. Montain and Young contributed to study conception and design and revised the article for important intellectual content. All authors reviewed, edited, and made important intellectual contributions to the manuscript.

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ABSTRACT

Systemic immune function is impaired by sleep restriction. However, the impact of sleep restriction on local immune responses, and to what extent any impairment can be mitigated by nutritional supplementation is unknown.

Objectives: We assessed the effect of 72-h sleep restriction (2-h nightly sleep) on local immune function and skin barrier restoration of an experimental wound, and determined the influence of habitual protein intake ($1.5 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) supplemented with arginine, glutamine, zinc sulfate, vitamin C, vitamin D3 and omega-3 fatty acids compared to lower protein intake ($0.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) without supplemental nutrients on these outcomes. Secondary outcomes included sleepiness, cognition, marksmanship, markers of gut barrier damage, and exploratory analyses to identify predictors of skin barrier recovery.

Methods: Wounds were created in healthy adults by removing the top layer of ≤ 8 forearm blisters induced via suction, after adequate sleep (AS) or 48-h of a 72-h sleep restriction period (SR; 2-h nightly sleep). A subset of participants undergoing sleep restriction received supplemental nutrients during and after sleep restriction (SR+). Wound fluid was serially sampled 48-h post-blistering to assess local cytokine responses. Performance on a battery of cognitive assessment and marksmanship tasks were collected throughout the 72-h sleep restriction period.

Results: The IL-8 response of wound fluid was higher for AS compared to SR (area-under-the-curve, AUC_i (\log_{10}), 5.1 ± 0.2 and $4.9 \pm 0.2 \text{ pg} \cdot \text{mL}^{-1}$, respectively ($P=0.03$); and, both IL-6 and IL-8 concentrations were higher for SR+ compared to SR ($p<0.0001$), signifying a potentially enhanced early wound healing response. Skin barrier recovery was shorter for AS (4.2 ± 0.9 days) compared to SR (5.0 ± 0.9 days) ($P=0.02$), but did not differ between SR and SR+

($P=0.18$). As expected, participants were tired and experienced cognitive declines in response to the imposed sleep restriction. In linear regression models adjusting for age, BMI, race, ethnicity, energy intake and study group, omega-3 intake was associated with longer healing time ([$\beta \pm$ SE] per g/d: 0.70 ± 0.33 , $P=0.04$), and protein intake was associated with shorter healing time (per g/d: -0.02 ± 0.01 , $P=0.01$). AUCi of IL-8 (per logged pg/mL: -1.50 ± 0.43 , $P<0.001$) and MIP-1b (per logged pg/mL: -1.42 ± 0.50 , $P=0.01$) sampled from the wound sites were associated with shorter healing time. Participants were also generally slower and less accurate on measures of cognitive performance and marksmanship. No effects on markers of gut barrier damage were observed.

Conclusions: Relatively modest sleep disruption induces cognitive declines and delays wound healing. Supplemental nutrition may mitigate some decrements in local immune responses, without detectable effects on wound healing rate. Wound healing time may be influenced by omega-3 and protein intake, as well as wound cytokines (but not serum biomarkers). Additional research is warranted to further elucidate these findings.

Keywords: sleep deprivation; cytokines; immune function; wound healing; skin barrier recovery; marksmanship; cognition; gut function; stress; Army; military personnel; first responders

INTRODUCTION

Immune responsiveness is degraded by short-term sleep restriction. The effect is mediated by hypothalamic-pituitary-adrenal axis and sympathetic nervous system activation (35), and characterized in part by decrements in natural killer cell activity and interleukin-2 production and increased levels of circulating proinflammatory cytokines (15, 36, 84). Collectively these effects are thought to increase risk of illness and infection (15, 63), and impair wound healing. For example, the risk of acquiring the ‘common cold’ was approximately 4-fold higher in volunteers who slept less than 6 h per night, compared to those who slept more than 7 h per night, for seven days (15, 63); and, 42-h total sleep deprivation delayed initial recovery of the skin barrier following an experimental wound. As such, developing strategies for maintaining immune function is of interest to populations in which short-term sleep restriction is sometimes unavoidable, such as military and emergency service personnel.

Optimal immune function is dependent on nutrient availability and underlying nutritional status (58, 77). Clinical nutrition support guidelines for adults recommend enteral formulations containing arginine, glutamine, omega-3 fatty acids, and antioxidants for immune-enhancement and faster recoveries in patients undergoing major elective surgery (50, 54, 58, 77). For example, vitamin C plays an important role in collagen synthesis, fibroblast proliferation, capillary formation and neutrophil activity (76) while omega-3 fatty acids enhance T-cell and natural killer cell activity, and have been shown to reduce systemic inflammation (12, 60). Further, studies indicate that certain nutrients improve wound healing indices in healthy adults (4, 42, 89). For example, arginine contributes to collagen deposition and cellular growth, and impacts microcirculation by increasing the production of nitric oxide (11, 58, 89), while glutamine stimulates the proliferation of fibroblasts, subsequently contributing to wound closure (58). The

efficacy of nutrient interventions for modulating immune function and promoting healing in healthy individuals who are immune-compromised consequent to physical or cognitive stressors (e.g., sleep restriction) has received less attention.

The suction blister model is a useful tool for studying immune responsiveness of populations exposed to a variety of stressors and the efficacy of countermeasures to promote or enhance recovery. Traditionally, circulating blood-derived markers of immune function have been assessed to study the systemic immune response (15, 36, 84), but these markers do not fully characterize functional status (e.g., the ability to heal from a wound or defend against an infection) (27). In contrast, wound healing models directly assess functional status of the innate immune system (i.e., the ability to heal from a wound), and can also provide insight into the local pro-inflammatory response and tissue remodeling processes. The suction blister model is a wound healing model that allows study of the functional immune response to include immune response at a wound site along with skin barrier restoration as a proxy measure of wound healing rate. Our group has shown that this method is sufficiently reliable for assessing skin barrier restoration and local immune responsiveness of experimental skin wounds (72) (i.e., strong correlations were observed between the left and right arm in terms of skin barrier restoration rate and local cytokine response). Further, the method has been used in humans to study how stress affects in-vivo immune responsiveness (27, 39, 66). For example, skin barrier restoration was delayed by approximately one day following a 30-min adverse social interaction (i.e., verbal disagreement), compared to a 30-min positive social interaction, with their spouse (39); and, college examination stress delayed suction blister wound healing time by approximately two days (66). In addition to demonstrating decrements in the immune response, delayed wound closure has practical implications, i.e., the potential for infection is heightened while the skin

barrier is perturbed. This is relevant for military trainees, wherein cellulitis and purulent skin abscesses are a common problem (38).

The primary aims of this study were two-fold. We first sought to demonstrate that the suction blister wound model is sensitive enough to detect decrements in local immune response and skin barrier restoration rate in response to a stress model (i.e., 72-h sleep restriction with 2-h nightly sleep in a laboratory environment), by examining effects of the stress model on skin barrier restoration. After successfully demonstrating acceptable sensitivity, we sought to determine if dietary supplementation with arginine, glutamine, vitamin C, vitamin D, zinc and omega-3 fatty acids could mitigate decrements in local immune response and skin barrier restoration. Secondary outcomes included sleepiness, cognition, markers of gut barrier damage, and an exploratory analyses to identify predictors of skin barrier recovery. We hypothesized that immune responses would be degraded, and skin barrier recovery would be delayed in participants following sleep restriction compared to free-living participants who were adequately rested. We further hypothesized that immune function would be preserved and skin barrier recovery would be shorter in participants who consumed 1.5 g protein per kg body weight (i.e., the higher end of the military dietary reference intake (MDRI), which was consistent with participants' habitual protein intake) and a twice-daily, multi-nutrient beverage during and after sleep restriction compared to participants who received a placebo beverage and 0.8 g protein per kg body weight (i.e., the low end of the MDRI). As a secondary objective, we examined the effects of sleep restriction on cognitive measures of attention and memory as well as marksmanship measures that included a friend-foe decision making task. Finally, a pilot study was conducted to identify associations between sleep restriction and markers of gut barrier

damage, and to determine the influence of multi-nutrient supplementation on gut barrier damage during sleep restriction.

MATERIALS AND METHODS

Study Design

This was a two-phase study. Phase 1 determined the effect of sleep restriction and controlled living conditions (i.e., residing in the laboratory) on local immune responses and skin barrier restoration. Phase 2 determined the effect of a nutrition intervention on local immune response and skin barrier restoration in response to sleep restriction under controlled living conditions. In phase 1, impact of short-term sleep restriction (i.e., ~72 hours of sleep restriction with 2-h sleep per night in a laboratory environment) on skin barrier restoration and immune response at the wound site was assessed by comparing free-living participants with adequate sleep (AS) to a group of sleep restricted participants who resided in the laboratory during the sleep restriction period (SR) (*Figure 1*). In phase 2, we determined if a diet providing 1.5 g protein per kg body weight combined with a multi-nutrient supplement during and after sleep restriction (SR+) attenuated the decrements in local immune function and skin barrier restoration observed in response to sleep restriction when compared with a diet providing 0.8 g protein per kg body weight combined with a placebo beverage (SR) (*Figure 1*). Dietary protein levels were selected to represent the lower and higher ends of the MDRI (0.8-1.6 g protein per kg body weight per day)(82), and the higher-level of 1.5 g protein per kg body weight per day was consistent with participants' reported habitual protein intakes. Blisters were applied to all three study groups (AS, SR and SR+), and the main measures of immune function included skin barrier restoration (measured by skin vapor permeability) and wound inflammatory responses.

Participants

Participants were military and civilian personnel assigned to Natick Soldier Systems Center, Natick, MA. Eighty-five percent (n = 56) of the 66 participants who began the study completed data collection and were included in the data analyses (AS, n = 16; SR, n = 20; and, SR, n = 20). One participant withdrew prior to study participation due to scheduling conflicts, seven volunteers withdrew during baseline testing (i.e., n = 3 due to relocation from the geographical area; and, n = 4 due to non-compliance with the sleep requirements leading up to the sleep restriction period), and three participants left the study during the sleep restriction period (i.e., SR, n = 2 due to gastrointestinal virus or migraine; and, SR+, n = 1 due to inability to stay awake).

Data collection occurred from September 2012 to May 2016 at the U.S. Army Research Institute of Environmental Medicine (Natick, MA). Each volunteer gave their written, informed consent after an oral explanation of the study. Individuals were included if they were between the ages of 19 and 35 years, were generally healthy and not taking medications (including non-steroidal anti-inflammatory drugs and aspirin), were not pregnant or lactating, had no history of psychiatric disorder requiring hospitalization or psychiatric medication usage, and slept between 7 and 9 hours per night at least five days per week. All subjects completed an initial screening and were medically cleared for participation. The study was approved by the Institutional Review Board, United States Army Research Institute of Environmental Medicine, Natick, MA. The investigators adhered to the policies for protection of human subjects as prescribed DOD Instruction 3216.02 and the research was conducted in adherence with the provisions of 32 CFR Part 219. The Clinicaltrials.gov identifier is NCT02053506.

Research procedures applicable to all experimental groups (AS, SR & SR+)

Assessment of General Sleep Patterns

Participants confirmed that they regularly slept 7-9 hours per night prior to the baseline testing period. General sleep patterns were assessed during the baseline testing period via the Morningness/Eveningness questionnaire, actigraphy, and a paper-and-pencil sleep diary. The Morningness/Eveningness questionnaire (32) is a 19-item questionnaire that assesses respondent's circadian preference, sleep-wake pattern for activity, and morning and evening alertness; and was used as an initial screener wherein participants needed to score between 31 and 69 to remain in the study, thus avoiding extremes in "morningness" or "eveningness". Participants wore an actigraphy monitor (Actical, Philips Respironics, Murrysville, Pennsylvania or an equivalent) for five days prior to the blister induction (AS) or live-in portion of the study (SR and SR+) to verify that they slept between 7 and 9 hours per night. Participants also maintained a paper-based sleep diary, in which they recorded the time they went to bed (with the intent to sleep) and the time they awoke.

Assessment of Life Stressors

The Perceived Stress Scale (16) was administered to all participants either within a week of the blister induction (AS) or upon arriving to the lab for the live-in portion of the study (SR and SR+) to assess life stressors in the previous month. This scale is a reliable and valid 14-item, widely used self-report measure of perceived stress, wherein respondents rate the stressfulness of their life during the previous month. The items are answered on a 0 (never) to 4 (very) scale, with higher sum scores indicating greater perceived stress.

Anthropometrics

Standing height was measured at baseline, in duplicate using an anthropometer (Seritex, Inc., Carlstadt, NJ or similar). Body weight was measured in shorts, t-shirt, stocking feet at baseline and either the morning of the blister induction (AS) or each day of the live-in portion of

the study (SR and SR+) using a calibrated electronic scale (Tanita WB-110A Class III, Tokyo, Japan).

Suction Blister Induction and Fluid Sampling

Venous blood was drawn from the forearm on the morning of the blister induction, and ~3.0 mL of serum was used to prepare an autologous fluid mixture to be used in the suction blister model (30% serum and 70% Hanks (+) buffer solution). CRP was assessed from serum and analyzed in duplicate using Multiplex bead based on Luminex® technology.

Suction blisters were induced according to previously described methods (72). Briefly, a vacuum pressure was applied to a polycarbonate template on the forearm to form a series of eight blisters (Figure 2). Blister fluid was subsequently sampled and the top of each blister was removed. Polycarbonate wells (Figure 2) were secured over the blisters and the autologous fluid mixture, which acts as a soluble chemotactic substance (90), was syringed into the polycarbonate wells. The concentration of inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α , MIP-1 α and MIP-1 β) was assessed by removing fluid from distinct wells at 4-h (AS, SR and SR+), 7-h (AS, SR and SR+), 24-h (AS, SR and SR+), and 48-h (SR and SR+) following blister formation.

Transepidermal Water Loss (TEWL) to Assess Skin Barrier Restoration

The time to skin barrier restoration was assessed by measuring TEWL from individual blisters using the VapoMeter (Delfin Technologies Inc., Stamford, CT). Beginning ~24-h after blister formation, TEWL was measured twice each morning, from the lower four wound sites and an adjacent, non-wounded, control site, and the paired measurements were averaged. If values were not within 10% of each other, a third measurement was taken and the two closest values were averaged. The TEWL measurements from wound number six were used to assess skin barrier restoration, since the majority of participants developed a blister at this location (i.e.,

all participants in AS and SR, and 18 of 20 participants in SR+) and our prior work indicated that blister size was consistent between participants at this site. A ‘Standard of Recovery’ was established using the TEWL values ~24 hours after blister induction (39):

$$[\text{TEWL measurement from wound site} - \text{TEWL measurement from control site (both measured ~24 hrs after blister induction)}] \times 0.10$$

The skin barrier was considered “restored” when a subsequent daily TEWL value (i.e., wound site measurement minus control site measurement) met or exceeded the ‘*Standard of Recovery*’. Participant’s daily TEWL values, from 24 hours post-blistering thru the day they reached the ‘*Standard of Recovery*’, were then exponentially regressed to better identify the precise moment of skin barrier restoration.

Additional Research Procedures Applicable Only to Sleep Restricted Participants (SR and SR+)

Participants underwent approximately 72 hours of sleep restriction with 2-h sleep per night in the laboratory to induce decrements in immune responsiveness and delay skin barrier restoration, and to identify if additional protein combined with a multi-nutrient beverage could mitigate these decrements (SR compared to SR+, Figure 1). Suction blisters were induced after 48-h of the sleep restriction protocol. The 72-h duration of sleep restriction with limited nightly sleep was selected based on the somewhat typical wake-restricted sleep that military personnel encounter during training (9) and combat missions ((49). The sleep-wake pattern in this study is also relevant to non-military emergency service personnel and medical interns, who may also encounter short-term scenarios where sleep restriction is unavoidable (5, 79, 85); and, endurance athletes who may self-impose sleep restriction during short-term, multi-day events (33, 43, 62).

Study participants arrived to the laboratory the day before the sleep restriction period began, and slept overnight at the laboratory. During the ~72 hour sleep restriction period,

participants slept only 2 hours per night and engaged in a variety of activities to maintain wakefulness (e.g., exercise, video games, television, movies), similar to the activities that were performed by participants in the free-living group that received adequate sleep (AS).

Determination of Total Daily Energy Expenditure

Total Energy Expenditure (6) during the sleep restriction period was estimated to determine the level of energy intake required to maintain body weight for each participant during the nutrition intervention experiment. TEE was estimated from approximate time spent sleeping, participating in miscellaneous activities (e.g., eating, watching TV, playing video games, personal care, moving about the dorm area, etc.), and exercise (67).

Physical Activity

The purpose of including mild to moderate physical activity during the sleep restriction period was to maintain wakefulness and sustain the participants' habitual level of energy expenditure. As such, exercise energy expenditure (EEE; $\text{kcal}\cdot\text{d}^{-1}$), derived from exercise recall interviews and added to the TEE equation, was used to determine the amount of mild to moderate physical activity that participants performed during the sleep restriction period. Exercise consisted of treadmill walking, outdoor walking and cycle ergometry. The American College of Sports Medicine's Metabolic Equations for steady state exercise conditions were used to estimate exercise workloads and subsequent energy expenditure (59). Trained study staff confirmed that all physical activity was performed at light intensity (self-reported using the 20-point Borg RPE scale (8)).

Assessment of Dietary Intake

Intake of omega-3 fatty acid-rich foods, probiotics and other dietary supplements (including multi-vitamin/minerals) was assessed at baseline by questionnaire, along with oral

antibiotic use; and, participants were asked to refrain from consuming these items for the duration of the study. Participants recorded all foods and beverages consumed for 3 days prior to each sleep restriction period, and for 5 days following each sleep restriction period to quantify intake of energy and macronutrients, as well as nutrients affecting immune function. Food records were reviewed daily and finalized for accuracy by Registered Dietitians, and analyzed for nutrient content using computer-based nutrient analysis software (Food Processor, ESHA Research, Salem, OR).

Study Diet

During sleep restriction period (Figure 1): Participants consumed measured and provided diets designed to maintain energy balance. Study diets included commercially-available food items and water was allowed *ad libitum*. Diets were designed by Registered Dietitians to provide either $\sim 0.8 \text{ grams} \cdot \text{kg}^{-1} \text{ body weight} \cdot \text{day}^{-1}$, which is the low end of the MDRI (SR) or $\sim 1.5 \text{ grams} \cdot \text{kg}^{-1} \text{ body weight} \cdot \text{day}^{-1}$, which is the higher end of the MDRI (SR+). The higher level of protein was chosen for the intervention diet based on general recommendations for immune-supporting diets, since proteins are a vital component of collagen synthesis (11, 54, 58, 77). Some of the food items provided by the study diet were chemically analyzed (Covance Inc., or equivalent) to confirm their composition of macronutrients and select micronutrients (i.e., vitamin C, vitamin D, n-3 fatty acids and/or zinc). Registered Dietitians prepared each participant's daily meals and snacks, and food consumption was monitored by trained study staff. Dietary intake was analyzed for nutrient content using computer-based nutrient analysis software (Food Processor, ESHA Research, Salem, OR). Participants were instructed to refrain from caffeine three days prior to the sleep restriction period to avoid the effects of caffeine

withdrawal during the sleep restriction period, and were not allowed to consume any other food or beverages other than those provided.

Post-sleep restriction period (Days 4-8, Figure 1): upon leaving the lab on Day 4, participants were instructed to consume a protein-controlled (SR: ~ 0.8 grams \cdot kg $^{-1}$ body weight \cdot day $^{-1}$; SR+: 1.5 grams \cdot kg $^{-1}$ body weight \cdot day $^{-1}$), *ad libitum* diet. Participants were given detailed instructions regarding protein-containing food, beverages, and portion sizes to meet the study's protein guidelines, and food records were reviewed daily by trained Registered Dietitians to confirm compliance.

Multi-Nutrient Beverage

The multi-nutrient beverage contained L-arginine (20 g \cdot d $^{-1}$), L-glutamine (30 g \cdot d $^{-1}$), omega-3 fatty acids (1 g \cdot d $^{-1}$), zinc sulfate (24 mg \cdot d $^{-1}$), vitamin D3 (800 IU \cdot d $^{-1}$) and vitamin C (400 mg \cdot d $^{-1}$). Content of the multi-nutrient beverage was based on formulas used in clinical settings which have shown benefits related to post-surgical infectious complications (10, 17, 18, 71, 73) and wound healing disorders (22). Nutrients were purchased from DSM Nutrition Products (Parsippany, NJ). The nutrient "pre-mix" (containing the arginine, glutamine, zinc, vitamin D and vitamin C) was added to an artificially sweetened, commercially available beverage powder using good manufacturing practices (4C Totally Light, Brooklyn, NY); and, the omega-3s (i.e., 500 mg docosahexaenoic acid, 300 mg eicosapentaenoic acid and 150 mg short-chain omega-3 fatty acids) were packaged separately. The placebo beverage was composed of the same commercially-available, artificially sweetened beverage powder (4C Totally Light, Brooklyn, NY) and 0.03 g of naringen and 0.004 g of quinine (both from Penta Manufacturing, Livingston, NJ) to impart a slightly bitter taste to match the taste of the 'multi-nutrient' beverage. The powders were stored in the refrigerator (omega-3 powder) or freezer ('multi-nutrient' and

placebo beverage powders); and, were added to containers and reconstituted with water prior to consumption. The beverages were consumed twice per day during the sleep restriction period (Days 1-3) and the post-sleep restriction period (Days 4-8). Study team members witnessed beverage consumption during the sleep restriction period and on each morning of the post-sleep restriction period; and, participants consumed the beverage, on their own, each afternoon of the post-sleep restriction period and returned the empty container the following morning.

Sleepiness and Cognition

Cognitive decrements subsequent to even short duration sleep loss or restriction have been well documented (45, 48, 88). In the present study, a short battery of cognitive tests focusing on executive control, working memory, and visual sustained attention was administered to quantify functional impacts performance (cognitive in this case)during the imposed sleep restriction. The Stanford Sleepiness Scale was administered immediately before the cognitive test battery to assess alertness. Participants completed the following cognitive tests at baseline (Day 1) and at approximately 0100 each morning of the sleep restriction period (Day 2 – Day 4):

- *Go/No-go*, a task module from the Automated Neuropsychological Assessment Metrics, Version 4, General Neuropsychological Screening Battery (13, 14), is a computer-assisted test of sustained attention and response control, wherein the participant must respond to a specific stimulus (i.e., pressing a button as quickly as possible when an “X” appears on the computer screen) and inhibit that action in response to other stimuli (i.e., no response when an “O” appears on the computer screen). A total of 120 trials were presented, including 96 target and 24 distracter stimuli, in a pseudorandom order.
- *Psychomotor Vigilance Task* (PVT) measured the speed with which the participant responded to a visual stimulus by pressing a button as soon as the light appears (i.e., light appears

randomly every few seconds for ~5–10 minutes) (19). Reaction time and accuracy were the main outcome measures.

- A *visual n-back test* was administered to evaluate attention and working memory capacity. This computer-based task, adapted from Kirchner (41) and McAllister and colleagues (53), was developed for use in this study from the simple continuous performance task module of the ANAM4 GNS battery (CSRC, 2013a,b). Participants were required to determine whether each number in a sequence matched a specified target number (0-back) or a number presented immediately prior (1-back) or 2 stimuli back (2-back) in the sequence. Numbers were presented at a rate of 1 every 3 seconds, with a total of 20 numbers presented in each n-back condition. Response accuracy and reaction times were recorded.

Systemic Markers of Inflammation and Immune function

Whole blood was drawn from a forearm vein daily, upon waking, during the live-in portion of the study. Cortisol, growth hormone, CRP, and cytokines were assessed from serum. Cytokines and CRP were measured, in duplicate, using Multiplex bead based on Luminex® technology. Cortisol and growth hormone were measured using the Immulite immunoassay system (Siemens Healthcare, Erlangen, Germany). Vitamin C and 25-hydroxyvitamin D were measured from blood on the morning of day 1 before sleep restriction to determine background micronutrient status, using colorimetric (BioVision, San Francisco, CA) and enzyme linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) respectively.

Leukocyte Migration of Wound Fluid

In a subset of SR+ (n = 4) and SR (n = 8) participants, suction blister wound exudate cells were characterized via flow cytometry. Following centrifugation of autologous wound fluid, exudate cells were re-suspended in FACS buffer (DPBS/2mM EDTA + 10% heat inactivated FBS) and incubated with Human Fc Receptor Binding Inhibitor (eBioscience) to reduce non-

specific binding. Characteristic light scattering properties and fluorescently labeled cell surface markers were used to quantify monocytes and polymorphonuclear cells (PMN; see *Figure 3* for gating strategy). The following anti-human primary fluorophore-conjugated antibodies, along with isotype controls, were used to characterize blister wound exudate leukocytes: FITC-conjugated anti-human HLA-DR, PE-conjugated anti-human CD16, PerCP-conjugated anti-human CD14, and APC-conjugated anti-human CD45 (all from eBioscience). Flow cytometry was performed on an Accuri C6 (BD Biosciences), and data were analyzed and figures generated using Cytobank (44).

Marksmanship Tasks

Marksmanship tasks were conducted on the EST 2000 (Cubic Corporation, Orlando, FL), with the session beginning at 3 h, 20h, 44 h and 68 h into sleep restriction and required approximately 90-120 minutes to complete. The EST 2000 is a small arms training system providing visual, auditory, and physical sensations mimicking the firing characteristics of an actual weapon via an M4 carbine adapted for use on the system. The modified weapon emits a Class 1 laser at a screen 30 m away from participants, providing information back to the trainer.

Two marksmanship tasks were implemented: a friend vs. foe discrimination task with varying levels of cognitive load and a modified Army Record Fire task. Each friend vs. foe task consisted of four, 16 min challenges: two low cognitive load (LCL) and two high cognitive load (HCL) challenges, each consisting of 80 targets and 8 cues. Twenty red or black E-silhouette targets appeared randomly at each of four locations (150 and 250 m; slightly left or right off center in each firing lane); ratios of black:red and friend:foe targets were presented 1:1. Targets were presented for 5 sec (dropping if hit, staying up for full 5 sec if missed) with a variable inter-target delay of 3-15 sec. At the start of each challenge, a brief (3 sec) visual cue indicated the

color criteria (red or black) that designated a target as friend or foe. For the LCL challenge, the color criteria remained constant with the black and red targets presented alternately. For the HCL challenge, targets were presented randomly, and the color criteria changed 8 times per challenge with a visual cue presentation. LCL and HCL challenges alternated, the starting challenge type was randomly chosen, and each challenge was followed by a 5 min rest period.

Up to four participants were tested simultaneously with the same challenge, but the meaning of cues differed for each firing lane depending on instructions given to individuals. Participants used a standing foxhole supported firing position and fired as quickly and accurately as possible at foes, taking only one shot per target, and pressed a button located directly above the trigger well as quickly as possible for friends. When a sequence of two consecutive same colored targets followed by two consecutive targets of the other color appeared in the HCL challenge, a button press was required on the fourth target, or high value target (HVT), in addition to the standard shot or button press. After each response, participants re-positioned weapons to the center of the firing lane, interrupted the sight picture, and waited for the next target to appear.

The EST 2000 recorded the latency to shoot at a foe or press the button for a friend and the accuracy of hits. The exact location of a bullet strike from the center of mass (Cartesian coordinates: 0,0) on the target was recorded, which allowed for calculating additional measures such as distance to the center of mass (DCM) and shot group tightness. The percentage of correct decisions (shot for a foe and button press for a friendly target) and number of HVTs detected/falsely detected was also calculated.

Following the friend vs. foe discrimination tasks, participants completed an Army Record Fire marksmanship protocol (United States, 2003) adapted to EST 2000 at USARIEM. Similar

to a live fire Record Fire qualification task, participants engaged targets (50-300 m) from prone supported (20 targets), prone unsupported (10 targets), and kneeling (10 targets) firing positions. The number of hits and misses for each target were recorded.

Markers of gut barrier damage

Archived serum derived from fasted morning samples collected by forearm venipuncture on the first (day 1) and final (day 4) mornings of each live-in phase were used to assess markers of gut barrier damage. Claudin-3 is a transmembrane tight junction protein involved in regulation of paracellular intestinal permeability, and is considered a marker of intestinal tight junction damage and paracellular intestinal barrier integrity (29). Claudin-3 was measured by ELISA according to manufacturer instructions (MyBiosource). Intestinal fatty acid binding protein (IFABP) is a small cytosolic protein expressed in enterocytes of the jejunum, and to a lesser extent the colon. IFABP is released into circulation upon enterocyte membrane integrity loss, and is considered a marker of intestinal epithelial cell damage (29). IFABP was measured by ELISA according to manufacturer instructions (Hycult Biotech). Lipopolysaccharide (LPS) binding protein (LBP) is an acute phase protein released from the liver in response to bacterial LPS (70), and is considered an indirect marker of bacterial LPS translocation from the gut lumen. LBP was measured by ELISA according to manufacturer instructions (Cloud Clone Corp).

Calculations and Statistical Analyses

The primary dependent variable of interest was skin barrier restoration rate, and secondary variables of interest were cytokine concentrations from the wound fluid. Mean and variance data from our prior work (72) were used for sample size calculations, and indicated that 20 participants were required to detect a 0.75 day (or 15%) difference in skin barrier restoration

time between groups ($\alpha = 0.05$, power = 0.80). Sample size calculations using 24-hr concentrations of IL-8, IL-6 and TNF- α from our prior work (72) indicated that ~20 participants were required to detect a ~40% difference in cytokine response ($\alpha = 0.05$, power = 0.80).

Area-under-the curve (AUC) values were calculated for each participant using data obtained from autologous wound fluid sampled following blister formation (i.e., wound cytokines concentrations). Briefly, “Area under the curve with respect to the increase” (AUC_i) (64) represents the total AUC_i for all measurements with consideration for the time difference between each measurement and their distance from the baseline value. In the few cases ($n = 3$) when no autologous wound fluid was available at the designated time-point(s) due to leakage from the well(s), the group mean was substituted in place of the missing value to calculate AUC_i. In cases where values were either more than 3 SDs from the mean or below the limits of detection, either the group mean or zeros, respectively, were substituted to calculate AUC_i.

Statistical analyses were conducted using the IBM SPSS statistical package version 19.0 (IBM Inc., Armonk, New York) and SAS version 9.4 (SAS Institute Inc., Cary, NC). Data were examined for outliers both quantitatively and graphically, and normal distribution of data was confirmed via the Shapiro-Wilk test. Data that were not normally distributed (i.e., cytokine serum and wound concentrations, CRP and GH) were log transformed (log₁₀). Repeated measures analysis of variance (ANOVA) was used to assess changes in body weight over time. Independent samples t-test was used to determine differences between AS and SR, and SR and SR+ for skin barrier restoration rate, cytokine concentrations from post-blister wound fluid (AUCs), and baseline measures (i.e., MEQ and PSS scores, CRP, 25-hydroxyvitamin D status, vitamin C status, and average sleep). The study was not powered to compare AS versus SR+, because this comparison was not of interest. Additionally, linear mixed models with first order

autoregressive covariance type was used to determine main effects of time and condition, and their interactive effects with regard to cytokine concentrations in blister wound fluid and serum, and GH, CRP and GH concentrations. Effects of time, multi-nutrient supplementation, and their interaction on markers of gut barrier damage were analyzed using linear mixed models adjusted for age and BMI. For all models, when significant main effects or interactions were observed, all possible t-tests were conducted and the Bonferroni correction was used to control the familywise error rate. Lastly, for the exploratory analyses to identify predictors of skin barrier recovery, multivariate linear regressions were used in models with all wound cytokines simultaneously, all serum biomarkers simultaneously, or all dietary variables simultaneously (plus energy intake), adjusting for age, BMI, study group, race and ethnicity. Results are presented as mean (\pm SD), unless otherwise noted. A two-tailed *P* value of 0.05 was considered statistically significant.

RESULTS

Baseline Demographics & Measurements

Baseline demographics are presented in Table 1, e.g., eighty-seven percent of study participants were male ($n = 52$) with an average age of 22 years. The dietary intake survey confirmed that participants did not habitually consume omega-3 fatty acid-rich foods, probiotics or other dietary supplements prohibited by the study protocol. There were no significant differences in any baseline characteristics or measures between AS and SR or SR and SR+, with the exception of higher 25-hydroxyvitamin D concentrations for SR compared to SR+ (Table 1).

Effect of sleep restriction on local inflammation and skin barrier restoration (AS versus SR)

Time to skin barrier restoration was significantly higher for SR (5.0 ± 0.9 days) compared to AS (4.2 ± 0.9 days, $P=0.02$). The analysis was repeated without females, to ensure that the sex imbalance between groups wasn't a confounder, and results were unchanged (AS, 4.1 ± 1.0 , and

SR, 5.0 ± 0.9 , $P=0.02$). These results confirmed the usefulness of the suction blister model for detecting immune function decrements in response to sleep restriction and controlled living conditions. This finding provided rationale supporting Phase 2, to determine if a nutrition intervention could mitigate immune function decrements in response to sleep restriction under controlled living conditions. Cytokine values from 13% of the 288 wells (AS and SR combined) were excluded from calculations, since less than 70% of autologous serum added to the chambers immediately post-blistering was recovered from these wells at the follow-on time-points. Wound fluid concentrations of IL-6, IL-8, MIP-1 α , MIP-1 β and TNF- α significantly increased over time for both AS and SR (*Figure 4*). A group x time interaction was observed for IL-8 ($P<0.0001$), wherein the mean concentration was higher for SR compared to AS at 7-h ($\log_{10} 2.6 \pm 0.4 \text{ pg}\cdot\text{mL}^{-1}$ and $\log_{10} 2.3 \pm 0.3 \text{ pg}\cdot\text{mL}^{-1}$, respectively, $P=0.004$); and, IL-8 concentration over the total sampling period (i.e., $\text{AUC}_{\log_{10}}$) was significantly higher for AS compared to SR ($5.1 \pm 0.2 \text{ pg}\cdot\text{mL}^{-1}$ and $4.9 \pm 0.2 \text{ pg}\cdot\text{mL}^{-1}$, respectively, $P=0.03$). No other significant between group differences were detected (*Figure 4*).

Effect of a multi-nutrient beverage on cognition, local and systemic inflammation, skin barrier restoration, marksmanship and gut barrier function in response to sleep restriction (SR versus SR+)

Anthropometrics, Energy Expenditure & Dietary Intake

Average TEE for SR and SR+ was $2860 \pm 410 \text{ kcals}\cdot\text{d}^{-1}$ and $2820 \pm 610 \text{ kcals}\cdot\text{d}^{-1}$, respectively ($P=0.8$), with EEE contributing $470 \pm 170 \text{ kcals}\cdot\text{d}^{-1}$ and $460 \pm 200 \text{ kcals}\cdot\text{d}^{-1}$ to TDEE, respectively ($P=0.9$). Body weight was not significantly different over time within or between groups during the sleep restriction period ($P=0.5$ and $P=0.6$, respectively), indicating that participants were in energy balance. There were no differences in dietary intake between SR and SR+ prior to the 72-hr live-in periods (Table 2). Per the study design, dietary intake of

protein (i.e., total grams, grams per kg body weight and percent of total energy intake), arginine, glutamine, omega-3 fatty acids, vitamin D, vitamin C and zinc were significantly higher for SR+, compared to SR, during and after the sleep restriction period (Table 2). Vitamin A was also significantly higher for SR+ compared to SR ([mean difference \pm SE] 512 ± 91 IU, $P < 0.0001$), during the sleep restriction period, due to higher intake of cheese products in the prescribed study diet. Daily energy intake was [mean difference \pm SE] 585 ± 146 kcals lower for SR compared to SR+ during the 5-d follow-up period ($P < 0.0001$).

Multi-nutrient Beverage

Compliance with the beverage prescription during and after the sleep restriction period was 100% and 99.7%, respectively (i.e., one participant in SR+ reportedly forgot to consume the beverage on the afternoon of Day 7, thus consumed only half of the daily dose of nutrients provided by the beverage on that particular day).

Sleepiness and Cognition

For both SR and SR+, participants were more significantly alert on day 1 compared to all other time points, as indicated by Stanford Sleepiness Scale (Day 1: SR, 1.7 ± 0.6 and SR+: 1.5 ± 0.6 ; Day 2: SR, 3.2 ± 1.1 and SR+: 3.0 ± 1.4 ; Day 3: SR, 3.8 ± 1.4 and SR+: 3.9 ± 1.3 ; Day 4: SR, 4.0 ± 1.6 and SR+: 3.4 ± 1.5) (*Figure 5*). There was no significant between group differences in terms of alertness scores (*Figure 5*).

Results for the go/no-go task indicated that participants in SR were significantly less accurate the last night of sleep restriction compared to all other time points (Day 1: $91.1 \pm 4.2\%$; Day 2: $91.8 \pm 5.2\%$; Day 3: $93.5 \pm 2.5\%$; Day 4: $86.2 \pm 9.8\%$) (*Figure 5*). There was a trend for a group-by-time interaction ($P=0.06$), wherein SR+ had (or tended to have) better accuracy compared to SR on the first ([mean difference \pm SEM] $3.4 \pm 1.8\%$, $P=0.07$), second ([mean

difference \pm SEM] $3.3 \pm 1.8\%$, $P=0.07$) and third ([mean difference \pm SEM] $5.7 \pm 1.8\%$, $P=0.002$) night of the sleep restriction period (*Figure 5*). There was no significant group or time effects with regard to mean response time (data not shown).

With regard to the PVT, reaction time was faster on day 1 compared to all other time points for SR and SR+, $p<0.01$ (*Figure 6*). A group effect indicated that reaction time was faster for SR compared to SR+ ([mean difference \pm SEM] -0.2 ± 0.01 seconds, $P=0.01$), but no group-by-time interactions were noted. Participants were generally more accurate at baseline and on the first night of the sleep restriction period compared to later time-points (*Figure 6*), with no between group differences or group-by-time interactions detected.

Accuracy and response time for 0-back, 1-back and 2-back conditions of the N-back task are shown in (*Figure 7*). Independent of group, significant time effects for the 0-back ($p<0.0001$) and 2-back ($P=0.03$) tasks indicated that accuracy was lower the last night of the sleep restriction period ([mean estimate \pm SEM] 0-back: $97.4 \pm 0.4\%$ and 2-back: $71.3 \pm 2.9\%$) compared to baseline ([mean estimate \pm SEM] 0-back: $99.9 \pm 0.4\%$, $p<0.0001$ and 2-back: $80.6 \pm 2.9\%$, $P=0.04$) and the first night of sleep restriction ([mean estimate \pm SEM] 0-back: $99.2 \pm 0.4\%$, $P=0.013$ and 2-back: $79.7 \pm 2.9\%$, $P=0.03$). With regard to response time, a significant time effect ($P=0.04$) indicated that participants tended to be slower for the 1-back task on the last night of sleep restriction ([mean estimate \pm SEM] 635 ± 19 milliseconds) compared to baseline ([mean estimate \pm SEM] 570 ± 19 milliseconds, $P=0.06$) and the first night of sleep restriction ([mean estimate \pm SEM] 574 ± 19 milliseconds, $P=0.06$). There were no significant between group differences with regard to 0-back, 1-back or 2-back accuracy or response time.

Systemic Markers of Inflammation and Immune function

For both SR and SR+, serum GH concentrations on day 1 were significantly lower, and circulating cortisol concentrations were significantly higher, compared to all other time points *Figure 8*. No within group changes over time were detected for CRP. A group x time interaction ($p < 0.0001$) was detected for cortisol which tended to be higher, or was significantly higher, in SR compared to SR+ on the morning of day 2 ([mean difference \pm SEM] 2.1 ± 1.2 $\mu\text{g/dL}$ and, $P = 0.07$) and day 4 ([mean difference \pm SEM] 4.7 ± 1.2 $\mu\text{g/dL}$, $p < 0.0001$), respectively *Figure 8*.

There were no within group changes for SR and SR+ in terms of IL-1 β , IL-6, IL-8, MIP-1 α and MIP-1 β serum concentrations; however, TNF- α serum concentration significantly declined from day 3 (\log_{10} $1.253 \text{ pg} \cdot \text{mL}^{-1}$) to day 4 (\log_{10} $1.156 \text{ pg} \cdot \text{mL}^{-1}$, $P = 0.002$) for SR (*Figure 8*). Although there was a main group effect ($p < 0.05$), wherein serum concentrations were higher for SR compared to SR+ with regard to IL-8 (Days 1-4, $p < 0.0001$), TNF α (Day 1 and Day 2, $P = 0.05$; and, Day 3, $P = 0.03$), and MIP-1 β (Days 1-4, $p < 0.0001$), there were no significant group-by-time interactions for any of the measured serum cytokines (*Figure 8*).

Immune response of autologous wound fluid

Cytokine values from 10% of the 320 wells (SR and SR+ combined) were excluded from calculations, since less than 70% of autologous serum added to the chambers immediately post-blistering was recovered from these wells at the follow-on time-points. Autologous wound fluid concentrations of IL-1 β , IL-6, IL-8, MIP-1 α , MIP-1 β and TNF- α significantly increased over time for both SR and SR+, with significant time x group differences (*Figure 9*). Additionally, AUCi concentrations were higher for SR+ compared to SR with regard to IL-6 (\log_{10} , 5.7 ± 0.3 $\text{pg} \cdot \text{mL}^{-1}$ and 5.3 ± 0.3 $\text{pg} \cdot \text{mL}^{-1}$, respectively, $p < 0.0001$) and IL-8 (\log_{10} , 6.3 ± 0.5 $\text{pg} \cdot \text{mL}^{-1}$ and 5.6 ± 0.1 $\text{pg} \cdot \text{mL}^{-1}$, respectively, $p < 0.0001$) (*Figure 9*). Results were not changed when baseline

25-hydroxyvitamin D concentrations, vitamin A intake during the sleep restriction period or energy intake during the 5-day recovery period were used as covariates.

In order to characterize blister wound exudate cells, flow cytometry was performed on cells isolated from the autologous wound fluid of a subset of SR+ (n = 4) and SR (n = 8) participants. During a pilot study, we were unable to isolate an adequate number of lymphocytes and macrophages from wound fluid (data not presented); thus, our analyses were restricted to monocytes and PMNs. The contribution of monocytes (i.e., CD14⁺CD16⁻) to the total pool of CD45⁺ lymphocytes decreased significantly over time and was similar between SR+ and SR groups (*Figure 10*). As expected, PMNs were the predominant blister wound exudate cell type; however, significant differences were observed with respect to PMN CD16 expression between SR+ and SR groups. At 48hrs, the proportion of CD16^{hi} PMNs was significantly reduced in SR+ participants compared to SR, whereas the proportion CD16^{lo} was significantly increased (*Figure 10*).

Skin Barrier Restoration

Time to skin barrier restoration was not significantly different between SR (5.0 ± 0.9) and SR+ (4.6 ± 0.8 days), P=0.18; and, results were unchanged when baseline 25-hydroxyvitamin D concentrations, vitamin A intake during the sleep restriction period or energy intake during the 5-day recovery period were used as covariates.

Predictors of Skin Barrier Restoration

Descriptive statistics are presented in terms of healing time tertiles, i.e., the time it took for skin barrier to restore to 90% (*Table 3*). In linear regression models of potential nutritional predictors of healing time, adjusted for age, BMI, race, ethnicity, energy intake and study group, omega-3 fatty acid (beta ±SE per g/d: 0.73 ±0.32 days, P=0.03) and zinc (per g/d: 0.10 ±0.05

days, $P=0.04$] intakes were associated with longer healing time; and, protein intake (per g/d: -0.02 ± 0.01 days, $P=0.01$) was associated with shorter healing time (*Table 4*). Calcium was subsequently removed from the model, since it was highly and significantly correlated with zinc, vitamin D and protein (Pearson $r = 0.7-0.8$); and both omega-3 and protein intake associations remained, while the association of zinc was attenuated: omega-3 intake: beta \pm SE per g/d: 0.70 ± 0.33 , $P=0.04$; protein intake per g/d: -0.02 ± 0.01 , $P=0.01$.

Incremental area under the curve values (AUCi) of IL-8 (beta \pm SE per logged pg/mL: -1.50 ± 0.43 , $P<0.001$) and MIP-1b (per logged pg/mL: -1.42 ± 0.50 , $P=0.01$) sampled from the wound sites were associated with shorter healing time (*Table 5*). None of the serum biomarkers were significantly associated with healing time (*Table 6*).

Marksmanship

Performance on marksmanship tasks changed during the testing period and, depending on the measurement, was affected by task condition, time, and/or challenge order. Changes in reaction times to engage a target were dependent on whether participants were responding to friends or foes. Main effects of time ($F_{3,42} = 11.51$, $P < 0.001$) revealed trigger pull reaction times when engaging foe targets slowed by average by 8% across each of the test periods relative to the initial testing period (*Figure 11*). In contrast to the latency to shoot a foe, there were significant main effects of task condition ($F_{1,14} = 22.02$, $P < 0.001$), challenge order ($F_{1,14} = 10.65$, $P=0.0057$), and time ($F_{3,42} = 15.15$, $P < 0.001$) on the latency to signal friendly targets (*Figure 12*). Participants were ~16% faster to signal friendly targets during the LCL condition compared to the HCL condition and, collapsed across condition, ~37% faster during the 3 h time point compared to the 68 h time point. Post hoc analysis revealed reaction times to signal friendly targets were significantly slower at the 44 and 68 h time points compared to the 3 h time

point as well as the 68 h time point compared to the 20 h time point. Collapsed across challenge condition and time, the main effect of challenge order revealed that participants were ~6% faster during the first task administration compared to the second.

Correctly discriminating between targets (shots for foe or button presses for friendly targets) and identifying the HVT also changed throughout the study. A significant time by challenge order interaction ($F_{3,42} = 4.22$, $P=0.011$) showed participants generally responded less frequently to targets over time. Omitted responses were, therefore, removed when calculating the percentage of correct responses to targets. With these omissions excluded, a significant interaction between task condition and time ($F_{3,42} = 5.32$, $P=0.0034$; *Figure 13*) was found, and a post hoc analysis revealed that under the HCL, but not LCL, condition, participants made significantly more errors (shooting a friendly target and pressing the button for a foe target) at the 44 and 68 h test points compared to the 3 and 20 h test points. For percentage of correct HVT detections (*Figure 13*), there were significant effects of challenge order ($F_{1,14} = 6.034$, $P=0.028$) and time ($F_{3,42} = 16.79$, $P < 0.001$). Participants successfully detected a higher percentage of HVTs during the first HCL challenge ($54\% \pm 6.5$) each day compared to the second ($45\% \pm 5.6$). In addition, HVT detections were higher at the 3 h time point compared to all other time points and at the 20 h time point compared to the 68 h time point. There were no significant effects on the number of HVT false detections.

Analysis of the qualification scores on the Army Record Fire task revealed a main effect of time ($F_{3,24} = 2.87$, $P=0.047$), with scores generally improving over time. Post hoc analysis did not show a significant change at any particular assessment point; however, qualification scores tended to be higher at 44 h compared to 3 h into the study. On the friend vs. foe task, the percentage of foe targets accurately hit did not change over time or by challenge order but was

affected by task condition ($F_{1,14} = 11.49$, $P=0.0044$; Figure 13), such that accuracy was higher during the LCL condition ($77\% \pm 2$) compared to the HCL condition ($75\% \pm 2$). There were no significant effects for shot placement, including average shot DCM, average shot group DCM, or the average radius of the shot group.

Gut barrier damage

No main effects of time, multi-nutrient supplementation or their interaction on any marker of gut barrier damage was documented (*Table 7*).

DISCUSSION

In this investigation we confirmed our initial hypothesis that skin barrier restoration was delayed for participants who underwent 72-h of sleep restriction with 2-h of sleep per night in a laboratory compared to participants who were adequately rested, with some degradation in cytokine response at the wound site during the initial phases of wound healing. Our second hypothesis was partially confirmed, wherein concentrations of pro-inflammatory cytokines at the wound site were higher during the initial phase of wound healing for participants who consumed habitual protein intake and a twice daily multi-nutrient beverage compared to participants who received a lower protein intake with placebo beverage during and after 72-h of sleep restriction with 2 h sleep per night. However, within the sleep-restricted groups we were unable to detect differences in skin barrier recovery in response to the nutrition intervention.

Expectedly, participants were tired and demonstrated degraded performance on measures of executive control, working memory, and visual sustained attention in response to the imposed sleep restriction, which is consistent with the literature (45, 48, 88). The finding that sleep restriction in a laboratory environment delayed skin barrier recovery, compared to free-living, adequately rested participants, is consistent with studies that investigated the impact of chronic

and acute psychological stress on wound healing (3, 28, 39, 40, 52). Further, Altemus et al. (2001) suggested that skin barrier function was perturbed after 42-h of total sleep deprivation, however, authors only measured skin barrier recovery within 3 hours following tape stripping (i.e., up to 75% recovery). Using the suction blister model, Kiecolt-Glaser et al. (2005) reported that skin barrier restoration was delayed by ~1 day following a 30-min adverse social interaction (i.e., verbal disagreement), compared to a 30-min positive social interaction, with their spouse. Roy et al. (2005) similarly reported that college examination stress delayed suction blister wound healing time by ~2 days. Taken together, findings from the current study and past investigations confirm that stressors, including sleep restriction, ultimately delay healing of an experimental wound. That decrements in wound IL-8 concentration over the total sampling period in the present study were lower in participants who underwent sleep restriction in a laboratory compared to free-living participants who were adequately rested suggests that the delayed wound healing in response to sleep restriction in a laboratory may be attributed to perturbations in the inflammatory response during the critical early phases of wound healing, as has been previously suggested by studies of psychological stress (27, 39). However, decrements in the later phases of the wound healing cascade may also be responsible for delayed healing after sleep restriction which can be assessed in future trials by sampling wound fluid beyond 48-h post-blistering.

We did not detect any changes in circulating markers of immune function over the course of the sleep restriction period, compared to baseline concentrations, with the exception of a decline in serum TNF- α concentration from day 3 to day 4 of the sleep restriction period. Of note, circulating cytokines on the morning of “day 1” may not have been an accurate depiction of “baseline” concentrations given that participants spent the previous night in the laboratory (i.e., an unaccustomed environment) and were potentially anxious about the impending sleep

restriction and related study activities. In support, the baseline concentrations of IL-1 β and TNF- α in the current study were more than three-fold higher than the pre-sleep restriction values reported by Altemus et al (2001). Regardless, acute sleep loss, either partial or total, seems to produce differential results with regard to the peripheral inflammatory cytokine response with some studies showing an increased production of pro-inflammatory cytokines (3, 25, 30, 34, 37, 83, 84) and others reporting no change over time (1, 69, 74). Further, cytokine concentrations from peripheral blood are thought to provide a ‘snapshot’ of systemic immunity, but may not reflect local immune responses (27, 39).

We did not detect significant differences in skin barrier recovery in participants who consumed supplemental nutrients during and after the sleep restriction period compared to those who received a non-nutritive placebo. The failure of the nutrition intervention to affect skin barrier recovery was surprising, given the higher concentrations of pro-inflammatory cytokines at the wound sites during the initial phase of wound healing in participants who received the nutrition intervention relative to those who received the placebo. There are few studies that have tested the efficacy of nutrition interventions on wound healing outcomes in healthy adults. Williams et al. (2002) reported that collagen deposition, as measured by the content of hydroxyproline in a subcutaneously-placed catheter, was higher in healthy older adults who consumed a mixture of arginine (7 g), β -hydroxy- β -methylbutyrate (HMB, 3 g) and glutamine (14 g) versus placebo twice daily for 14 days. Two other studies supplemented the diets of healthy younger (~30 years) and older adults (~70 years) with 30 g/d of arginine and demonstrated higher collagen deposition at an experimental wound site and increased peripheral blood lymphocyte mitogenesis compared to placebo (4, 42). Those findings lead us to expect that the nutrition intervention would accelerate skin barrier restoration. However, another published

human study tested the efficacy of a nutrition intervention (i.e., 4 weeks of eicosapentaenoic/docosahexaenoic polyunsaturated fatty acid, PUFA, supplements) on skin barrier restoration and authors did not detect a significant difference between the experimental and placebo group (55). While the sleep restriction did cause delayed healing time in the current study, the effect size may not have been large enough to allow detection of the effects of the nutrition intervention.

We observed that under sleep-restriction, the nutrition intervention group experienced higher IL-6, IL-8 and MIP-1 β concentrations at the wound site during healing, compared to the placebo group, which potentially indicates an enhanced response during the early phases of wound healing. These findings are consistent with Martinez et al. (2004) and McDaniel et al. (2008) who also reported higher cytokine expression at a wound site (i.e., IL-1 β , with a trend for higher IL-6 and TNF- α , concentrations) within 24-h of blistering, with no significant differences in healing time in non-sleep restricted participants who were either supplemented with PUFA or placebo for 4 weeks. Although the functional significance of altered local cytokine concentrations in the present context remains unclear, increased expression of pro-inflammatory and chemotactic cytokines during the initial phase of wound healing is thought to be advantageous given their multifaceted roles that are expected to promote healing, e.g., enhancing phagocytosis, stimulating the migration of keratinocytes at the edges of the wound, promoting fibroblast chemotaxis and proliferation, and stimulating re-epithelialization, tissue remodeling and the formation of new blood vessels (21, 68, 87). Although, the decreased proportion of CD16 $^{+}$ PMNs in SR $^{+}$ participants at 48 hrs suggests that neutrophil apoptosis is accelerated in this group (20), elevated levels of IL-8 may account for this, as IL-8 has previously been shown to decrease human PMN expression of CD16 (51). Taken together, the somewhat equivocal

findings in the current study (i.e., potential benefits of the nutrition intervention under sleep restriction conditions were observed at the wound site while no significant effect on skin barrier restoration was detected) suggest that additional research is warranted to elucidate the functional implications of enhancing local immune responses.

Contrary to expectations, we did not detect an increase in cortisol concentrations or decline in growth hormone over the course of the sleep restriction period, as has generally been demonstrated in the literature in response to acute sleep restriction (7, 26, 35, 69). Similar to the current study's results, Altemus et al. (2001) reported no change in cortisol in response to 42-h of total sleep deprivation compared to baseline levels (6.1 ± 0.9 and 5.5 ± 0.9 $\mu\text{g/dL}$, respectively). Interestingly, participants had lower serum GH and higher serum cortisol concentrations on the first morning of the study, prior to the sleep restriction intervention, compared to subsequent time-points during the sleep restriction period. This is somewhat consistent with results reported by Vgontzas et al. (2004), who observed lower peak cortisol secretion after sleep restriction compared to baseline. Since normal variations in hormone concentrations (e.g., cortisol) occur across a 24-h period, it would be advantageous to sample circulating levels at multiple time-points throughout the day to further characterize the response and also to measure catecholamines. Additionally, measuring these hormones on the first morning of the study may not represent "baseline" concentrations as previously mentioned herein. Indeed, baseline cortisol concentrations in the present study were almost three-fold higher compared to the pre-sleep restriction values reported by Altemus et al. (2001). Of note, we detected lower cortisol concentrations after 24- and 72-h hours of sleep restriction in participants receiving the nutrition intervention compared to controls, i.e., cortisol declined in all participants after the initial day 1 sampling, but declined more for subjects who consumed additional protein and the multi-nutrient

810 beverage during and after sleep restriction, indicating the nutrition intervention seems to have
811 modulated the cortisol response under sleep-restriction conditions.

812 Our exploratory analyses investigating predictors of skin barrier restoration indicated that
813 omega-3 and protein intake may influence wound healing time under sleep-restriction conditions.
814 That we observed that habitual omega-3 fatty acid intake was positively associated with longer
815 healing time was somewhat surprising, given the role of omega-3 fatty acid intake in mitigating
816 the inflammatory response. However, this exploratory analysis was limited by the small sample
817 size and additional cases are needed to confirm these findings. Additionally, our analyses
818 indicated that wound healing time may be predicted by wound cytokines, but not serum
819 biomarkers. These findings allude to the functional implications of enhancing local immune
820 responses. The fact that serum biomarkers weren't predictive of skin barrier restoration was
821 unsurprising, given prior observations that cytokine concentrations from peripheral blood may
822 not reflect local immune responses (27, 39). Additional analyses with a larger data set is
823 warranted, which ultimately may inform future studies assessing nutritional countermeasures to
824 stress-related effects on healing and provide further evidence of mechanisms related to skin
825 barrier recovery.

826 With respect to marksmanship performance, results suggest that over a 72 h period of
827 obtaining only 2 h of sleep per night, Soldiers take significantly longer to make decisions, make
828 the wrong decisions regarding the identity of friends or foes, and do not believe their
829 performance is suffering. The longer latencies to fire observed in this study are consistent with
830 previous investigations (46, 57, 81). The degree to which reaction times degraded, however,
831 were not similar between friend or foe targets, such that latencies increased by only ~16% when
832 engaging foe targets but by ~37% when engaging friendly targets. It is unknown if this result is

833 due to differential decision-making between friend and foe targets, differences in motor
834 sequences and responses between the shot and the button press (for example, longer engagement
835 latencies to shoot a target may compensate for slower decision-making), or another factor.

836 Determining how participants made their decision in future studies may elucidate this question.

837 In contrast to reaction times, shooting accuracy and precision changed little throughout
838 the duration of the study, which conflicts with other studies that show decreased marksmanship
839 accuracy following sleep loss (31, 57). One explanation is that previous studies incorporated
840 additional forms of stress into their protocols, such as intense physical and/or mental training
841 outside of the testing period. These additive stressors may have degraded marksmanship
842 performance to a greater extent than sleep loss alone. Second, marksmanship methods vary
843 largely among studies. In contrast to the current study, which required participants to take a
844 relatively large number of shots over a short period of time, previous studies required
845 significantly fewer shots within a given time frame (31, 57). Third, given that participants in this
846 study were relatively new to military service, repeated exposures to the marksmanship tasks may
847 have improved performance simply through practice. Such improvements may explain the
848 modest increase in Record Fire scores observed in this study. Practice effects may also have
849 tempered the extent to which marksmanship latencies and friend versus foe discrimination
850 declined with sleep loss. Future research should continue to explore the relationship between
851 sleep and marksmanship, and identify potential mitigation strategies for the deleterious effects of
852 sleep loss.

853 Contrary to our hypothesis, sleep restriction did not alter circulating concentrations of gut
854 damage markers. Sleep restriction has been shown to activate a canonical stress response as
855 evidenced by increased HPA-axis activity and cortisol release, although this response has not

856 been observed in all studies (65), including this study. This stress response is thought to
857 contribute to intestinal cell injury and cell death, ultimately leading to inflammation and
858 disruption of the gut barrier (2, 23, 80). In support, sleep deprivation and circadian disruption
859 have been shown to increase intestinal permeability and facilitate bacterial translocation into
860 systemic circulation in murine models (24, 65, 80). The absence of a stress (i.e., cortisol)
861 response in this study may suggest that the study environment did not induce a stress response of
862 sufficient magnitude to degrade gut barrier integrity during sleep restriction. Alternately,
863 claudin-3 and IFABP may not be sensitive enough markers to detect subtle damage to the gut
864 barrier. Future studies should determine whether functional measures such as dual sugar
865 absorption tests may be more sensitive to effects of sleep restriction on gut barrier function.
866 Finally, the absence of changes in gut damage markers likely prevented detection of any effect of
867 multi-nutrient supplementation on gut barrier integrity despite evidence that several nutrients
868 including the glutamine, vitamins A, C and D, zinc, and omega-3 fatty acids have all been
869 independently shown to positively impact gut barrier health in prior studies (47, 56, 61, 78, 86).

870 This study presents limitations that should be considered in terms of data interpretation
871 and planning of future trials. We cannot exclude the possibility that differences in housing
872 conditions between AS and SR contributed to the observed differences between these groups.
873 However, any stressors experienced during the free-living AS phase would be expected to bias
874 results towards the null and living in the laboratory during the sleep restriction period was part of
875 the stressor in Phase 1 of the study. Additionally, greater-than-expected variability between
876 participants may have decreased our ability to detect differences between the sleep restriction
877 groups in terms of skin barrier restoration. Additionally, both groups reportedly consumed ~1.5
878 g protein per kg body weight leading up to the sleep restriction period, which was consistent with

the prescribed protein intake for SR+ but higher than the prescribed protein intake of SR, thus potentially confounding the results. Therefore, including a protein-controlled diet prior to the intervention to habituate liver enzymes to the prescribed protein intake is prudent. Lastly, while energy intake was different between the sleep restriction groups during the five day follow-up period, the estimated energy imbalance was $< 100 \text{ kcal} \cdot \text{d}^{-1}$ for three of the five days. Further, it is unlikely that a mild energy deficit for the remaining two days affected wound healing (77) and results were unchanged when energy intake was included as a covariate in the analysis. Despite these shortcomings, this study provided valuable insight into the local pro-inflammatory response and tissue remodeling processes, and nutritional interventions to support the innate immune system, during and after sleep restriction.

CONCLUSION

Herein, we demonstrate that the suction blister model is an effective model for testing the immune response to stressors and for testing the efficacy of countermeasures to mitigate immune decrements (e.g., nutrition interventions) during stress. Using this model we showed that 72-h of sleep restriction with 2-h sleep per night in a laboratory delays skin barrier recovery, thus underscoring the importance of adequate sleep when feasible. However, when adequate sleep is not feasible (e.g., military personnel in training/combat, emergency service personnel, ultra-endurance athletic competitions), these findings suggest that maintaining protein intake at the higher end of the MDRIs in combination with a multi-nutrient beverage (i.e., containing arginine, glutamine, zinc, vitamin C, vitamin D and omega-3 fatty acids) may attenuate some decrements in local immune responses observed during sleep restriction relative to a lower protein intake without nutrient supplementation, albeit without affecting skin barrier recovery. Additional research is needed to elucidate the functional implications of this improved local

immune response.

Conflict of Interest: *The authors declare that they have no competing interests*

Disclaimers

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the US Army or the Department of Defense. Any citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. Study funded by the US Army Medical Research and Materiel Command.

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Table 1. Baseline Characteristics for Study Participants

Baseline Data	Group			<i>P-value</i> AS vs SR	<i>P-value</i> SR vs SR+
	Adequate Sleep (n = 16)	72-h Sleep Restriction (n = 20)	72-h Sleep Restriction with nutrition intervention (n = 20)		
	[Mean \pm SD]				
Age	23.2 \pm 4.7	21.2 \pm 3.9	21.0 \pm 3.2	0.17	0.83
Gender					
Male	12	20	20		
Female	4	0	0		
Race					
Hispanic/Latino	2	3	8		
Not Hispanic/Latino	14	17	20		
Ethnicity					
Caucasian	14	13	16		
Black or African American	2	4	1		
Other	0	3	3		
BMI (kg/m ²)	25.7 \pm 3.7	26.5 \pm 3.8	26.2 \pm 3.8	0.51	0.78
MEQ (total score) ¹	56.2 \pm 6.4	52.7 \pm 3.7	54.7 \pm 4.2	0.06	0.11
Sleep (hrs/night) ²	7.99 \pm 0.50	7.71 \pm 0.67	7.64 \pm 0.50	0.18	0.71
PSS (total score) ³	28.4 \pm 3.1	29.2 \pm 5.8	29.8 \pm 4.6	0.62	0.72
C-reactive protein _{log10} (mg/L)	0.1 \pm 0.6	0.4 \pm 0.4	0.2 \pm 0.5	0.39	0.68
Vitamin C (umol/L)	N/A	28.9 \pm 15.4	37.4 \pm 14.6	N/A	0.84
25-hydroxyvitamin D (ng/ml)	N/A	24.7 \pm 6.7	19.2 \pm 6.1	N/A	0.01

¹Morningness Eveningness Questionnaire²Hours of sleep per actigraphy monitoring³Perceived Stress Scale

Table 2. Diet characteristics of participants who underwent 72-h sleep restriction without (SR) and with (SR+) multi-nutrient beverage

	72-h Sleep Restriction (SR) (n = 19)				72-h Sleep Restriction with nutrition intervention (SR+) (n = 20)			
	Pre-study	Live-in	Post-study	Live-in and post-study combined	Pre-study	Live-in	Post-study	Live-in and post-study combined
	← Mean ± SD →				← Mean ± SD →			
Energy (kcal)	2715±682 ^{ab}	2860±416 ^{cd}	1881±419 ^{ace}	2248±333 ^{bde}	3004±825 ^a	2817±594 ^b	2466±499 ^{***ab}	2598±494 [*]
Protein (g)	119±39 ^{abc}	65±9 ^a	72±12 ^b	70±10 ^c	134±48	120±23 ^{**}	121±22 ^{**}	121±22 ^{**}
Protein (g·kg ⁻¹ body weight)	1.5±0.5 ^{abc}	0.8±0.0 ^a	0.9±0.1 ^b	0.9±0.1 ^c	1.7±0.5	1.5±0.0 ^{**}	1.5±0.1 ^{**}	1.5±0.1 ^{**}
Protein (% of total energy)	17.8±5.2 ^{ab}	9.1±0.5 ^{acd}	16.0±3.3 ^{ce}	13.4±2.1 ^{bde}	17.9±4.0 ^a	17.1±0.9 ^{***b}	20.7±4.2 ^{***ab}	19.4±2.6 ^{**}
CHO (g)	320±98 ^{ab}	470±73 ^{acd}	248±78 ^{bce}	332±60 ^{de}	346±102 ^{ab}	414±96 ^{acd}	273±65 ^{bc}	326±68 ^d
CHO (% of total energy)	47.1±8.3 ^{abc}	65.6±2.2 ^{ade}	51.8±7.2 ^{bdf}	57.0±4.4 ^{cef}	46.9±8.2 ^a	58.5±2.0 ^{**abc}	43.4±4.6 ^{***bd}	49.1±2.9 ^{**cd}
Fat (g)	107±35 ^{abc}	82±13 ^{ad}	66±14 ^{bd}	72.2±9.8 ^c	116±37 ^{ab}	79±14 ^{ac}	98±23 ^{***c}	91±18 ^{***b}
Fat (% of total energy)	35.2±5.7 ^{ab}	25.9±2.3 ^{acd}	32.2±5.4 ^c	29.9±3.6 ^{bd}	34.1±5.0 ^a	25.4±1.8 ^{abc}	35.7±3.6 ^{*bd}	31.8±2.5 ^{cd}
Arginine (g)	3.8±2.3 ^{ab}	3.1±0.4 ^c	2.1±0.7 ^{ac}	2.5±0.6 ^b	4.5±2.4 ^{abc}	23.4±0.6 ^{***a}	23.3±1.3 ^{***b}	23.4±0.9 ^{***c}
Glutamine (g)	11.7±6.4 ^a	14.0±2.2 ^{bc}	6.6±2.2 ^{ab}	9.4±2.0 ^c	15.0±8.3 ^{abc}	45.8±2.7 ^{**ad}	40.6±3.9 ^{**bd}	42.6±3.1 ^{***c}
Omega 3 (g)	1.0±0.6 ^{ab}	1.8±0.4 ^{acd}	0.5±0.3 ^{bce}	1.0±0.2 ^{de}	1.3±0.6 ^{abc}	2.3±0.2 ^{**ade}	1.7±0.3 ^{***bd}	1.9±0.2 ^{***ce}
Vit A (IU)	6096±6941	1829±265 ^a	6393±4436 ^a	4682±2784	9849±13008 ^a	2341±312 ^{***a}	6517±6069	4951±3820
Vit D (IU)	143.0±99.1 ^{abc}	11.3±8.0 ^a	58.5±48.1 ^b	40.8±30.7 ^c	200.9±146.3 ^{abc}	815.2±4.8 ^{**ade}	951.2±99.9 ^{**bd}	900.2±62.8 ^{***ce}
Vit C (mg)	134.2±113.2 ^a	67.7±39.4 ^a	110.4±94.4	94.4±59.4	123.0±93.0 ^{abc}	448.0±36.4 ^{***a}	460.4±46.3 ^{**b}	455.8±35.5 ^{***c}
Zinc (mg)	10.0±4.8 ^{abc}	6.5±1.2 ^a	5.3±1.8 ^b	5.8±1.5 ^c	11.5±6.5 ^{abc}	31.0±1.0 ^{***a}	32.3±2.3 ^{**b}	31.8±1.6 ^{***c}

Independent samples t-test was used to determine differences in nutrient intake between SR and SR+ during each study period (i.e., indicated in the column headings); *indicates significant difference from SR, $p < 0.05$; **indicates significant difference from SR, $p < 0.0001$. ANOVA was used to determine within group differences between each study period (e.g., live-in and post-study). Similar superscript letters indicate significant within group differences for each nutrient ($p < 0.05$).

Table 3. Unadjusted means of characteristics

Characteristic	Healing Time Tertiles			P trend
	T1	T2	T3	
N	12	13	13	
Healing Time, d	3.9 (0.1)	4.7 (0.1)	5.8 (0.1)	0.00
Age (years)	23.2 (1.0)	21.0 (0.9)	19.6 (0.9)	0.01
Average sleep prior to sleep restriction	447.0 (10.1)	470.0 (9.7)	467.9 (9.7)	0.15
BMI (kg/m ²)	27.2 (1.1)	26.3 (1.1)	25.5 (1.1)	0.30
Body weight (kg)	84.4 (3.8)	79.8 (3.7)	76.7 (3.7)	0.15
Education, %				0.41
High school	41.7	61.5	53.9	
Some college	33.3	30.8	38.5	
Associate degree	25.0	0.0	7.7	
Master degree	0.0	7.7	0.0	
Race, % white	66.7	69.2	76.9	0.84
Hispanic, %	25.0	23.1	30.8	0.90
Perceived stress scale scores (pre-study)	29.1 (1.6)	30.2 (1.5)	29.3 (1.5)	0.93
Pre-Study Diet (3-day average)				
Calcium (mg)	1095.7 (134.7)	841.6 (129.4)	756.0 (129.4)	0.08
Carbohydrates (g)	343.3 (29.7)	306.5 (28.6)	343.2 (28.6)	0.98
Carbohydrates (% of total energy intake)	44.2 (2.4)	47.6 (2.3)	48.4 (2.3)	0.20
Fat (g)	121.0 (10.6)	101.8 (10.2)	113.1 (10.2)	0.62
Fat (% of total energy intake)	35.5 (1.5)	33.0 (1.5)	36.3 (1.5)	0.69
Energy intake (kcal)	3082.3 (224.9)	2663.5 (216.1)	2819.8 (216.1)	0.42
Omega 3 fatty acids (g)	1.2 (0.2)	0.9 (0.2)	1.3 (0.2)	0.66
Protein (g)	148.3 (12.5)	123.2 (12.0)	108.8 (12.0)	0.03
Protein (g·kg ⁻¹) body weight)	1.8 (0.2)	1.5 (0.1)	1.5 (0.1)	0.12
Protein (% of total energy intake)	19.6 (1.3)	18.8 (1.2)	15.4 (1.2)	0.02
Vitamin A (IU)	14186.2 (2864.0)	6302.3 (2751.6)	3651.8 (2751.6)	0.01
Vitamin C (mg)	144.6 (28.9)	90.6 (27.8)	136.9 (27.8)	0.88
Vitamin D (IU)	234.3 (36.1)	164.4 (34.7)	135.0 (34.7)	0.05
Zinc (mg)	12.2 (1.7)	10.8 (1.6)	9.5 (1.6)	0.26
Logged Serum Values				
Vitamin C (pg·mL ⁻¹ , baseline)	1.5 (0.1)	1.6 (0.1)	1.3 (0.1)	0.03

Vitamin D (pg·mL ⁻¹ , baseline)	1.3 (0.1)	1.3 (0.1)	1.3 (0.1)	0.92
Cortisol (pg·mL ⁻¹ , AUCi)	3.1 (0.1)	2.8 (0.1)	3.1 (0.1)	0.99
CRP (pg·mL ⁻¹ , AUCi)	3.06 (0.14)	2.80 (0.14)	3.05 (0.14)	0.99
GH (pg·mL ⁻¹ , AUCi)	3.00 (0.14)	2.76 (0.14)	3.02 (0.14)	0.90
IL-1B (pg·mL ⁻¹ , AUCi)	1.21 (0.12)	1.54 (0.12)	1.51 (0.12)	0.09
IL-6 (pg·mL ⁻¹ , AUCi)	4.01 (0.01)	4.00 (0.01)	4.00 (0.01)	0.11
IL-8 (pg·mL ⁻¹ , AUCi)	4.04 (0.02)	4.00 (0.02)	3.98 (0.02)	0.047
MIP-1a (pg·mL ⁻¹ , AUCi)	4.01 (0.03)	4.02 (0.02)	3.97 (0.02)	0.23
MIP-1b (pg·mL ⁻¹ , AUCi)	4.01 (0.01)	4.00 (0.01)	3.99 (0.01)	0.16
TNFa (pg·mL ⁻¹ , AUCi)	4.05 (0.03)	3.99 (0.03)	4.00 (0.03)	0.26
Logged Wound Values				
IL-1b (AUCi)	4.9 (0.2)	4.7 (0.2)	4.9 (0.2)	0.83
IL-6 (AUCi)	5.5 (0.1)	5.4 (0.1)	5.5 (0.1)	0.54
IL-8 (AUCi)	6.1 (0.1)	5.9 (0.1)	5.7 (0.1)	0.02
MIP-1a (AUCi)	5.2 (0.1)	5.2 (0.1)	5.2 (0.1)	0.65
MIP-1b (AUCi)	5.3 (0.1)	5.2 (0.1)	5.1 (0.1)	0.19
TNFa (AUCi)	4.3 (0.1)	4.2 (0.1)	4.4 (0.1)	0.60

Table 4. Relationship between pre-study diet variables and healing time

Parameter	Estimate	SE	P
BMI (kg·m ²)	0.04	0.04	0.32
Age (yrs)	-0.03	0.05	0.54
Energy (kcal)	0.00	0.00	0.15
Group A vs. B (ref.)	0.59	0.29	0.06
White vs. Non-White (ref.)	0.18	0.31	0.56
Hispanic vs. Non-Hispanic (ref.)	0.24	0.32	0.47
Ca (mg)	0.00	0.00	0.12
Ω3 Fatty acids (g)	0.73	0.32	0.03
Vit A (IU)	0.00	0.00	0.12
Vit C (mg)	0.00	0.00	0.49
Vit D (IU)	0.00	0.00	0.31
Zinc (mg)	0.10	0.05	0.04
Protein (g)	-0.02	0.01	0.01

Note: Beta coefficients and standard errors estimate using multivariate linear regression including all variables simultaneously

Table 5. Relationship between wound cytokines and healing time

Parameter	Estimate	SE	P
BMI (kg·m ²)	0.04	0.04	0.30
Age (yrs)	-0.08	0.04	0.06
Group A vs. B (ref.)	-0.75	0.47	0.12
White vs. Non-White (ref.)	0.19	0.36	0.60
Hispanic vs. Non-Hispanic (ref.)	0.12	0.31	0.70
LOG IL-1B (AUC)	0.53	0.58	0.37
LOG IL-6 (AUC)	-0.21	0.65	0.75
LOG IL-8 (AUC)	-1.50	0.43	0.00
LOG MIP-1a (AUC)	1.06	0.63	0.10
LOG MIP-1B (AUC)	-1.42	0.50	0.01
LOG TNF α (AUC)	0.33	0.80	0.69

Note: Beta coefficients and standard errors estimate using multivariate linear regression including all variables simultaneously

Table 6. Relationship between serum cytokines and healing time

Parameter	Estimate	SE	P
Intercept	66.59	98.85	0.51
BMI	-0.02	0.05	0.65
Age_yrs	-0.13	0.06	0.04
Group A vs B (ref.)	0.61	0.34	0.09
White vs. Non-White (ref.)	0.39	0.40	0.34
Hispanic vs. Non-Hispanic (ref.)	0.41	0.44	0.36
LOG Cortisol (AUC)	-0.39	0.34	0.27
LOG CRP (AUC)	-0.30	0.45	0.51
LOG GH (AUC)	0.08	0.44	0.85
LOG IL1B (AUC)	-45.49	24.63	0.08
LOG IL6 (AUC)	-1.21	17.16	0.94
LOG IL8 (AUC)	0.72	2.29	0.75
LOG MIP1 α (AUC)	44.81	22.81	0.06
LOG MIP1 β (AUC)	-2.20	6.92	0.75
LOG TNF α (AUC)	-10.92	8.65	0.22

Note: Beta coefficients and standard errors estimate using multivariate linear regression including all variables simultaneously

Table 7. Markers of gut barrier damage.

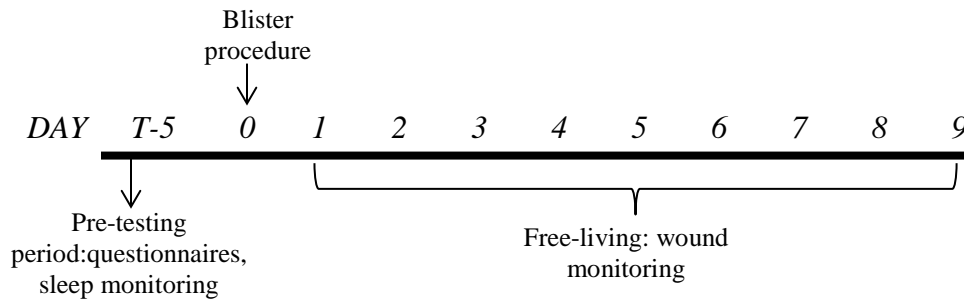
	SR (n=20)	SR+ (n=20)	P-value ¹		
			Group	Time	Interaction
Serum claudin-3 (ng/mL)			0.86	0.60	0.21
Day 1	5.3 ± 3.2	4.8 ± 3.1			
Day 4	4.8 ± 2.9	5.0 ± 3.2			
Δ	-0.4 [-0.9, -0.02]	0.2 [-0.8, 1.1]			
Serum IFABP (pg/mL) ²			0.09	0.22	0.41
Day 1	974 ± 414	1272 ± 902			
Day 4	918 ± 509	1204 ± 653			
Δ	-56 [-248, 135]	-69 [-371, 233]			
Serum LBP (μg/mL) ²			0.24	0.84	0.61
Day 1	7.4 ± 2.3	7.0 ± 2.2			
Day 4	7.7 ± 2.3	6.9 ± 1.9			
Δ	0.3 [-0.5, 1.1]	-0.1 [-1.4, 1.1]			

Values are mean ± SD or mean [95% CI]. IFABP, intestinal fatty acid binding protein; LBP, lipopolysaccharide binding protein; SR, sleep restriction; SR+, sleep restriction with multi-nutrient supplementation.

¹Linear mixed model adjusted for age and BMI.

²Log₁₀-transformed for analysis.

A



B

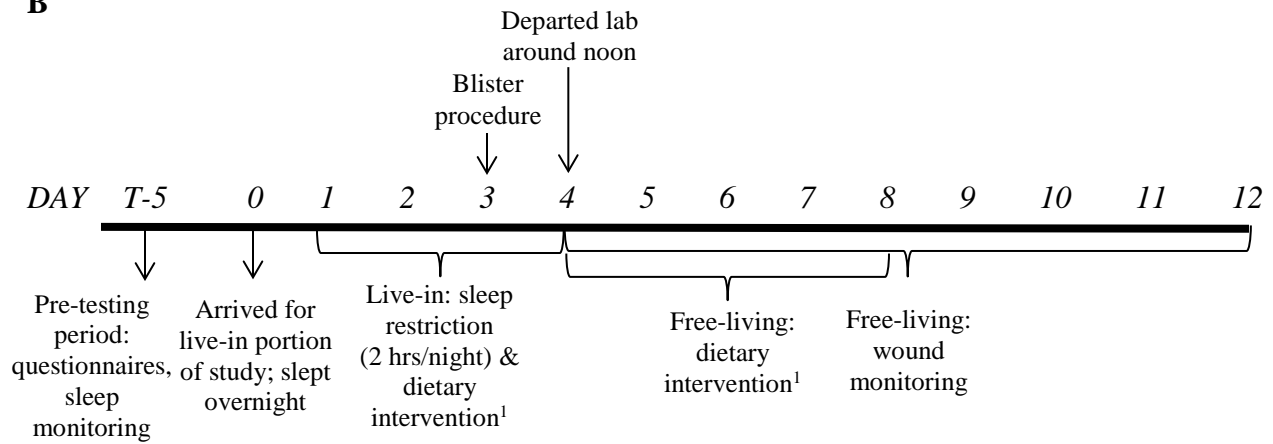


Figure 1. Timeline of activities during adequate sleep (**A**) and sleep restriction (**B**) phases.

¹Dietary intervention during sleep restriction consisted of 0.8 g protein/kg body weight plus placebo beverage (SR) or 0.8 g protein/kg body weight plus multi-nutrient beverage (SR+).



Figure 2. Photographs of the suction blister template, the subsequent blisters and the wound fluid collection template

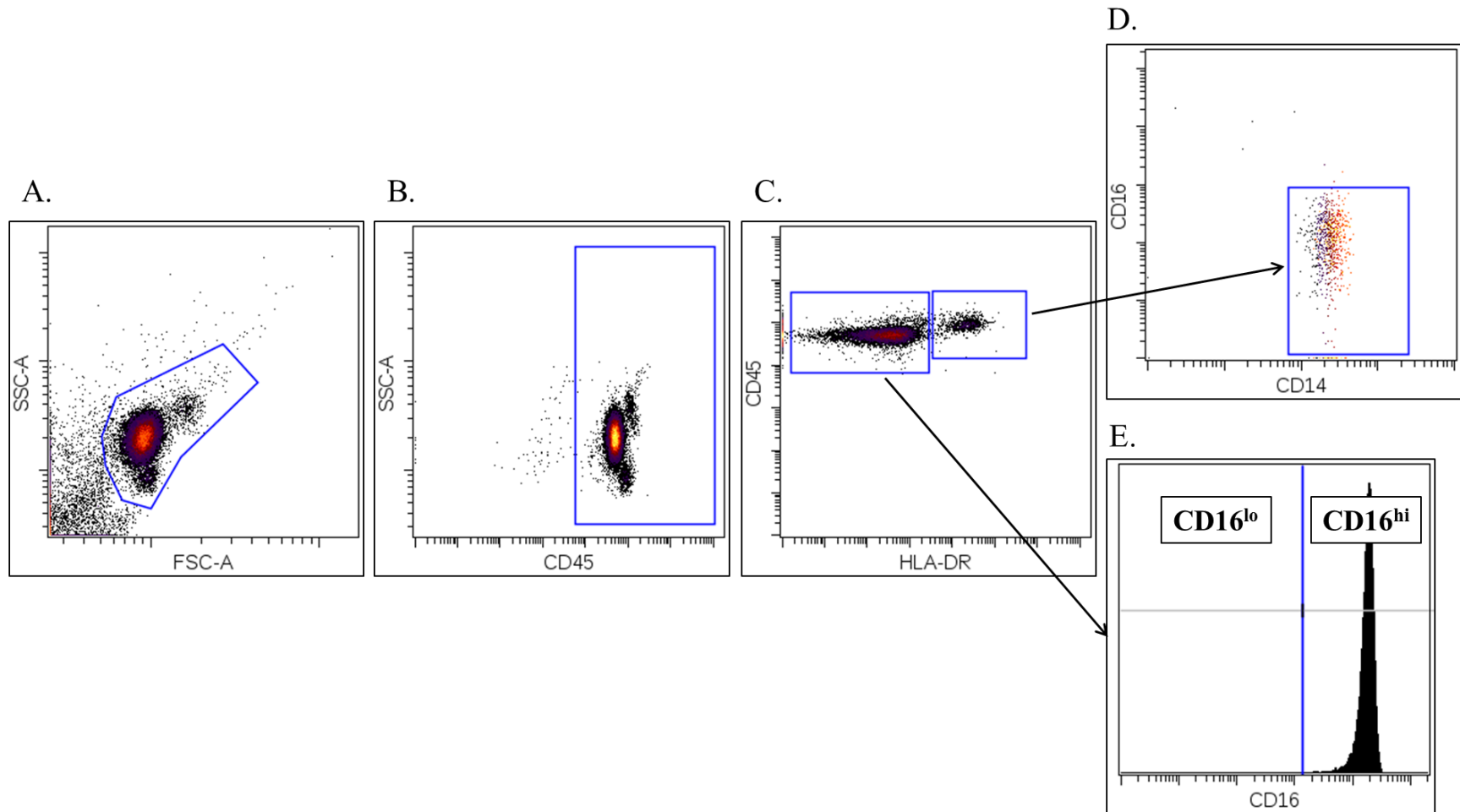


Figure 3: Flow cytometry gating strategy for blister wound exudate cells.

(A) Cells isolated from autologous wound fluid were first gated according to side and forward scatter. (B) CD45 was used to identify exudate leukocytes. (C) CD45⁺ cells were then examined for HLA-DR expression. (D) Monocytes were identified as HLA-DR⁺CD16⁻CD14⁺. (E) HLA-DR⁻ polymorphonuclear cells were further characterized by CD16 expression, and classified as either CD16^{lo} or CD16^{hi}.

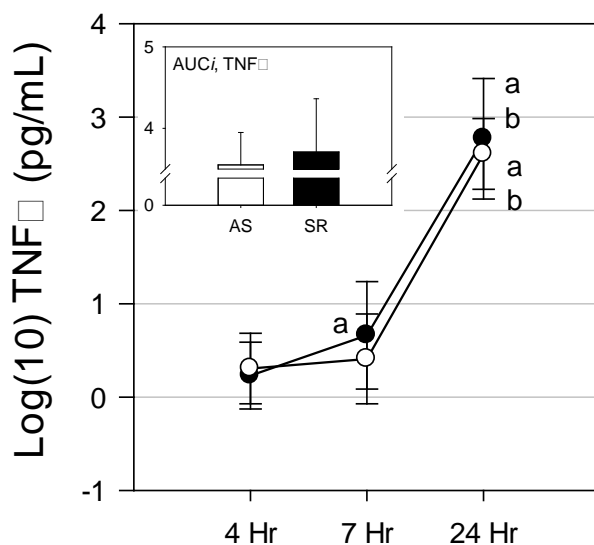
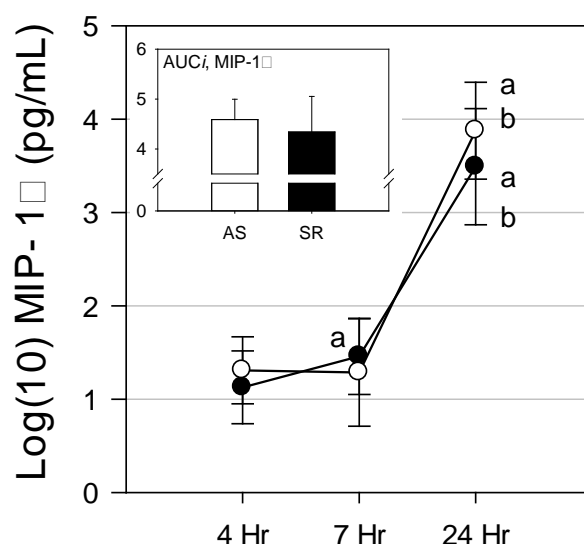
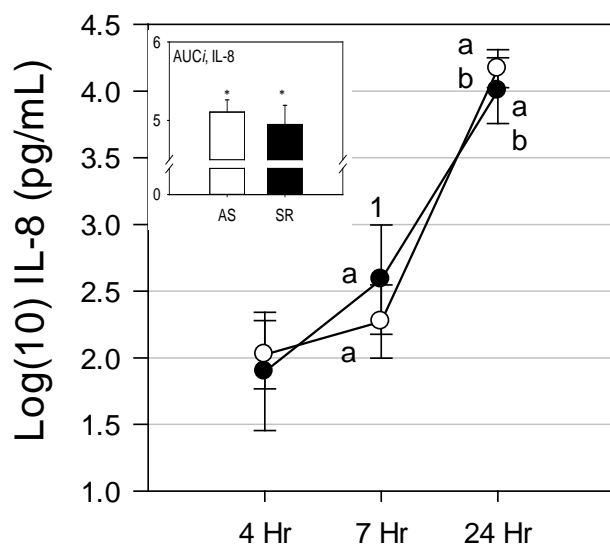
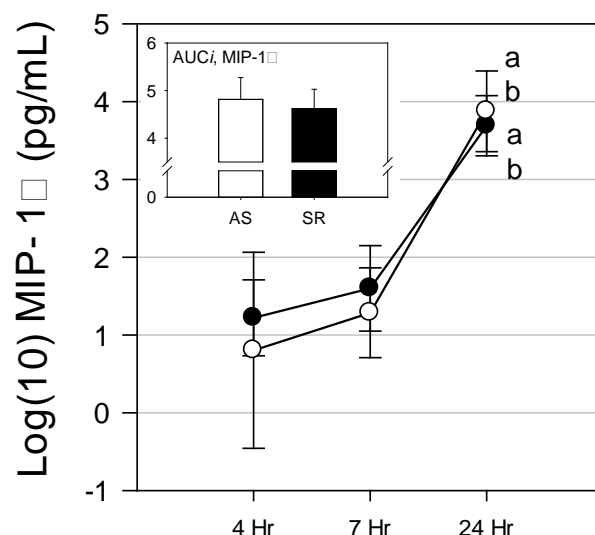
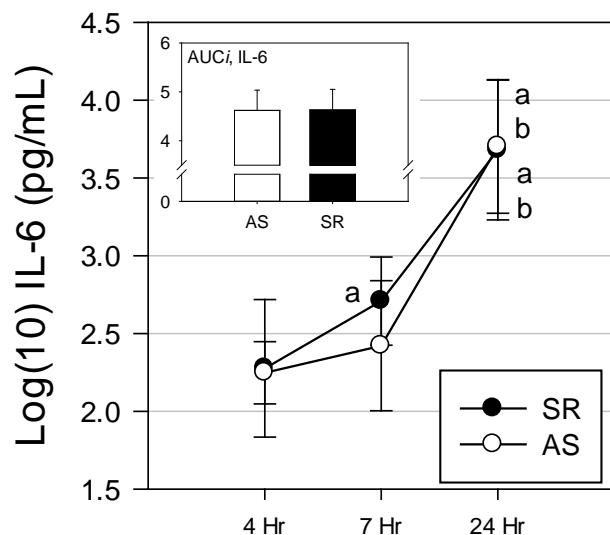


Figure 4: Cytokine response of wound exudate in participants who underwent adequate sleep (AS) compared to 72-h sleep restriction (SR)

AS = blisters were induced after 5 nights of adequate sleep (i.e., 7-9 hours of sleep per night confirmed via activity monitors); SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night). ^a = significantly different from 4 Hr time point ($p < 0.05$), ^b = significantly different from 7 Hr time point ($p < 0.05$), ¹ = indicates significant between group difference at specified time-point ($p < 0.05$). Values are means \pm SD.

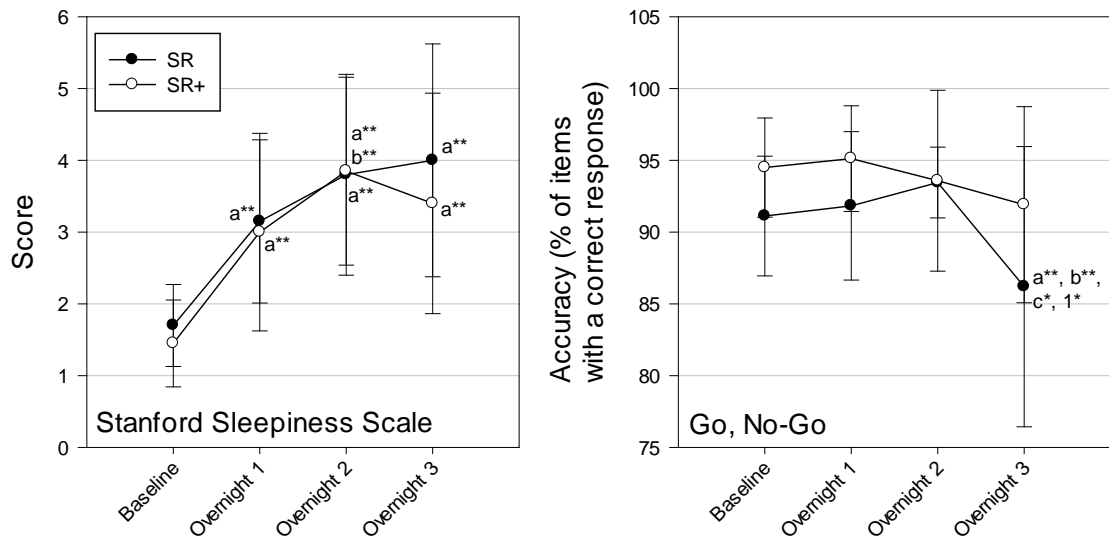


Figure 5: Stanford Sleepiness Scale Scores & Go, No-Go Accuracy

SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) without additional dietary protein or multi-nutrient beverage; SR+ = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) with additional dietary protein or multi-nutrient beverage.

^asignificant within group difference from day 1

^bsignificant within group difference from day 2

^csignificant within group difference from Day 3

¹significantly different from SR+

* $p < 0.05$, ** $p < 0.0001$

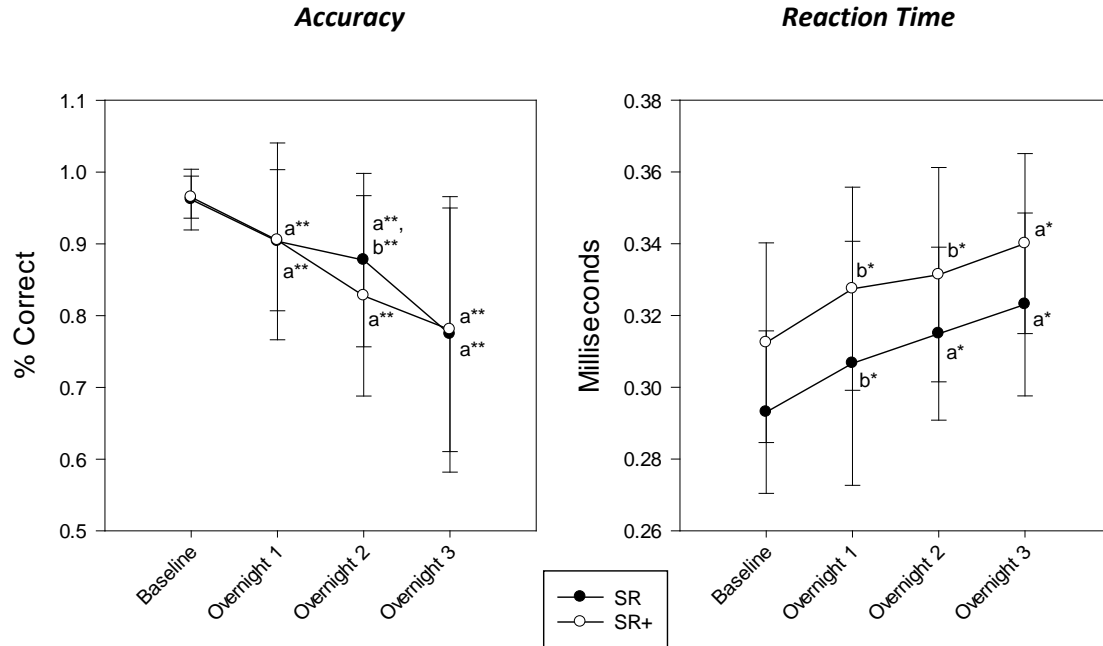


Figure 6: Psychomotor Vigilance Task: Accuracy and Reaction Time

SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) without additional dietary protein or multi-nutrient beverage; SR+ = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) with additional dietary protein or multi-nutrient beverage. ^asignificant difference within group from day 1, ^bsignificant difference within group from day 4, * $p < 0.05$, ** $p < 0.01$

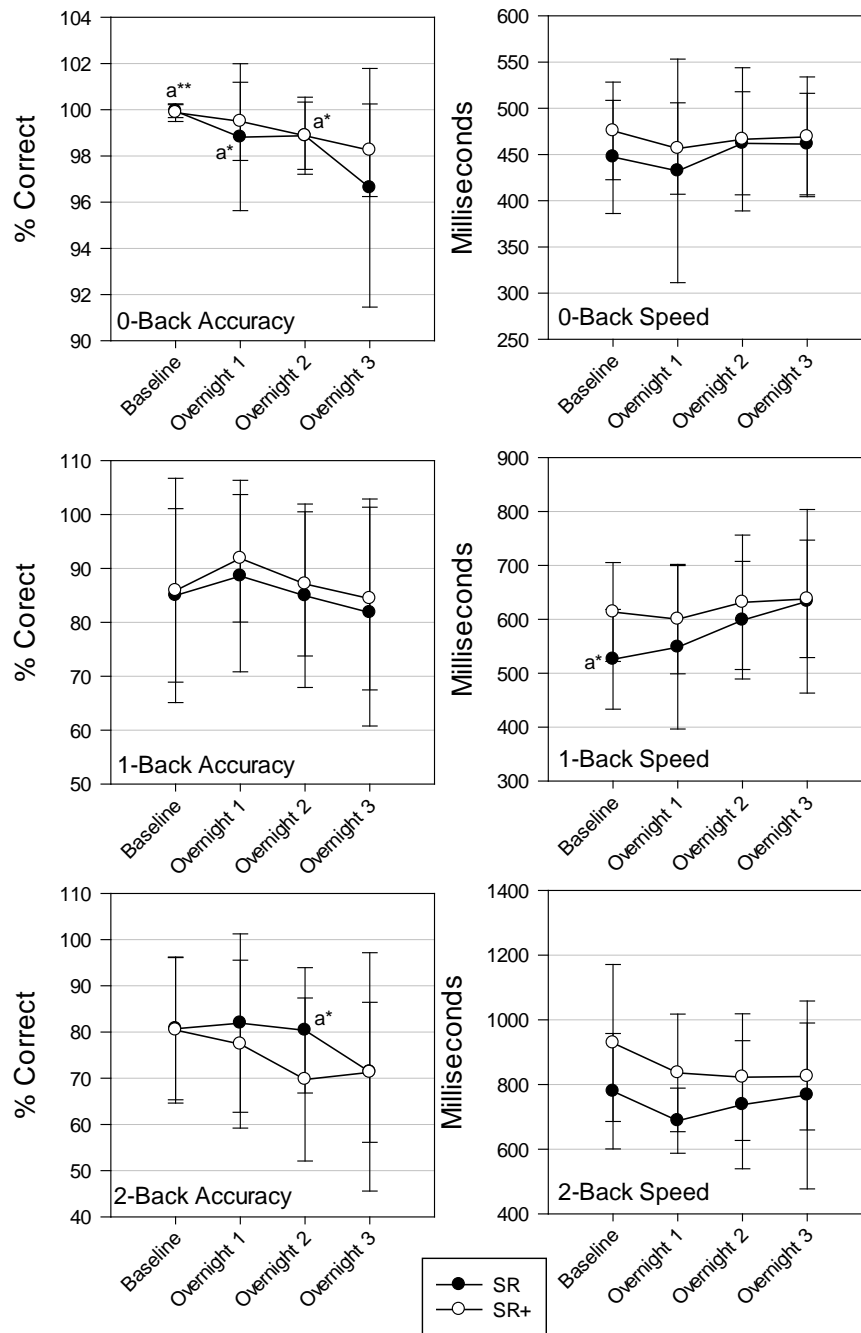


Figure 7: 0-Back, 1-Back, 2-Back Accuracy and Speed results.

SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) without additional dietary protein or multi-nutrient beverage; SR+ = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) with additional dietary protein or multi-nutrient beverage.

^asignificant difference within SR from day 4

* $p < 0.05$, ** $p < 0.0001$

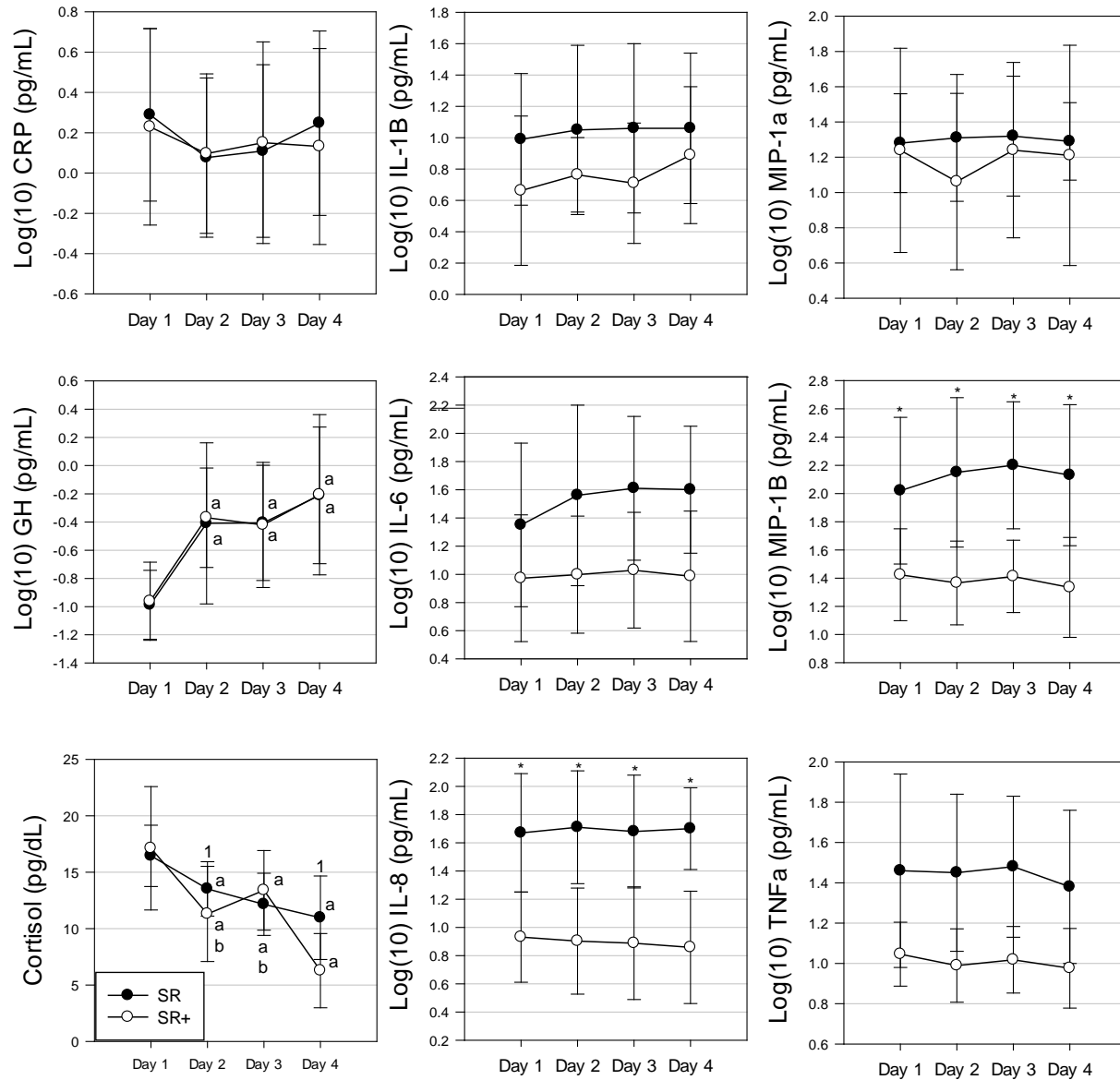


Figure 8: Circulating concentrations of serum C-Reactive Protein (CRP) Growth Hormone (GH), Cortisol, and Cytokines.

SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) without additional dietary protein or multi-nutrient beverage; SR+ = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) with additional dietary protein or multi-nutrient beverage. Linear mixed models with first order autoregressive covariance type was used to determine main effects of time and condition, and their interactive effects. When significant main effects or interactions were observed, all possible t-tests were conducted and the Bonferroni correction was used to

control the familywise error rate. Values are means \pm SD.^asignificant within group difference from Day 1 ($p < 0.05$); ^bsignificant within group difference from day 4 ($p < 0.05$); ^lsignificantly different from SR+ ($p < 0.05$), *indicate significant difference between groups at specified time-points ($p < 0.0001$).

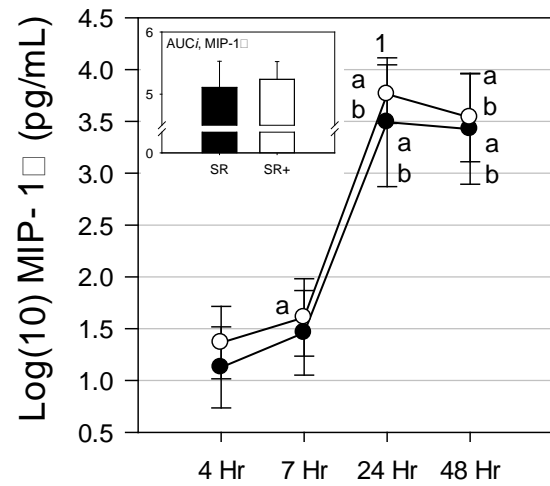
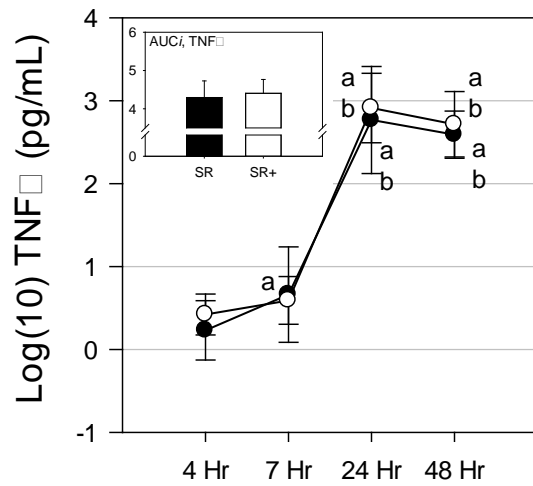
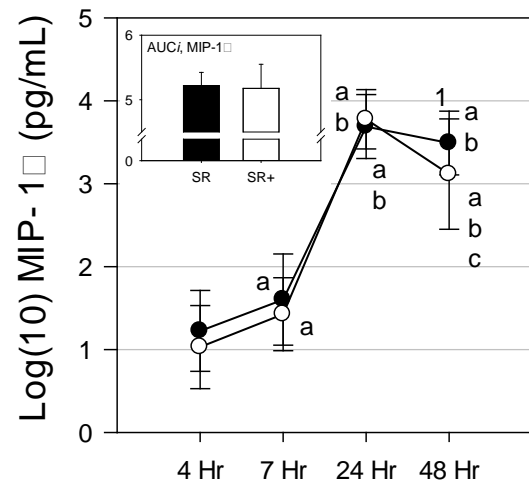
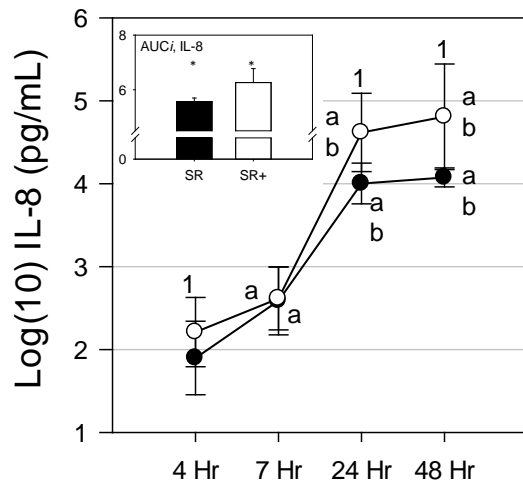
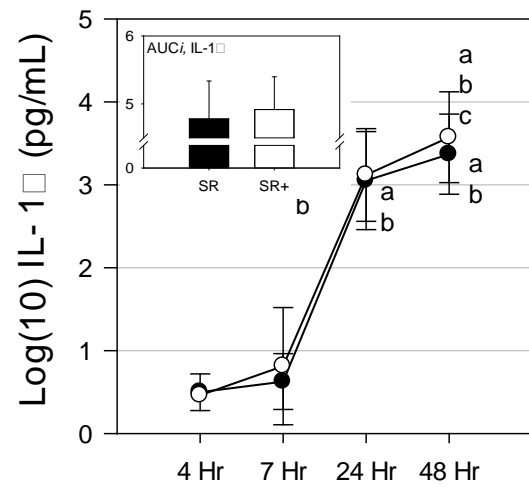
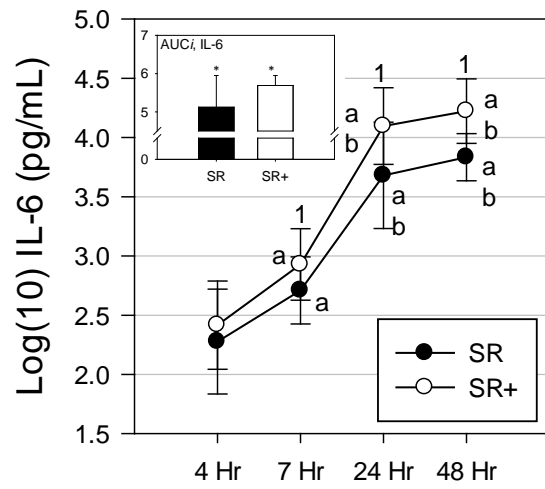


Figure 9: Cytokine response of wound exudate in participants who underwent 72-h sleep restriction with and without nutrition intervention.

SR = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) without additional dietary protein or multi-nutrient beverage; SR+ = blisters were induced after 48-h of sleep restriction in participants who underwent 72-h of total sleep restriction (monitored in laboratory with ~2-h sleep per night) with additional dietary protein or multi-nutrient beverage. ^a = significantly different from 4 Hr time point ($p < 0.05$), ^b = significantly different from 7 Hr time point ($p < 0.05$), ^c = significantly different from 24 Hr time point ($p < 0.05$), ¹ = indicates significant between group difference at specified time-point ($p < 0.05$). Linear mixed models with first order autoregressive covariance type was used to determine main effects of time and condition, and their interactive effects. When significant main effects or interactions were observed, all possible t-tests were conducted and the Bonferroni correction was used to control the familywise error rate. Independent samples t-test was used to determine differences between groups in terms of total cytokine concentrations (AUCs). Values are means \pm SD.*indicates significant differences between SR and SR+ in terms of AUCs.

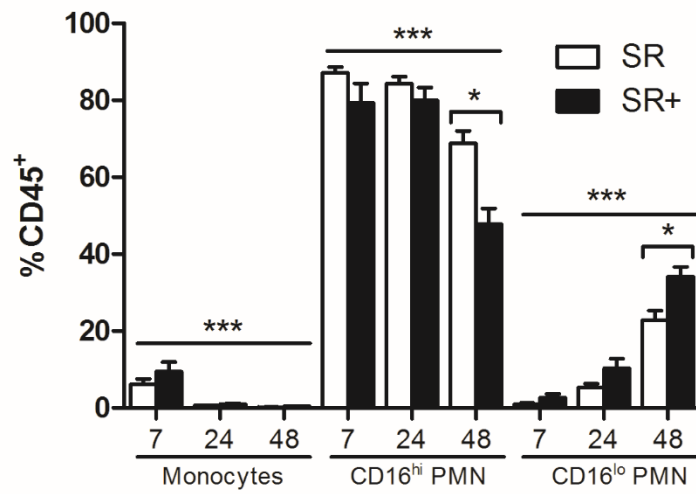


Figure 10: Blister exudate leukocyte populations. CD14⁺CD16⁻ Monocytes and CD16^{hi} and CD16^{lo} polymorphonuclear cells were isolated from the autologous wound fluid of SR and SR+ participants ($n = 8$ and 4 , respectively) at 7, 24, and 48 hours post blister induction. Data are expressed as percent of total CD45⁺ population \pm SEM. *** = $p < 0.001$ for time effect; * = $p < 0.05$ for SR vs. SR+

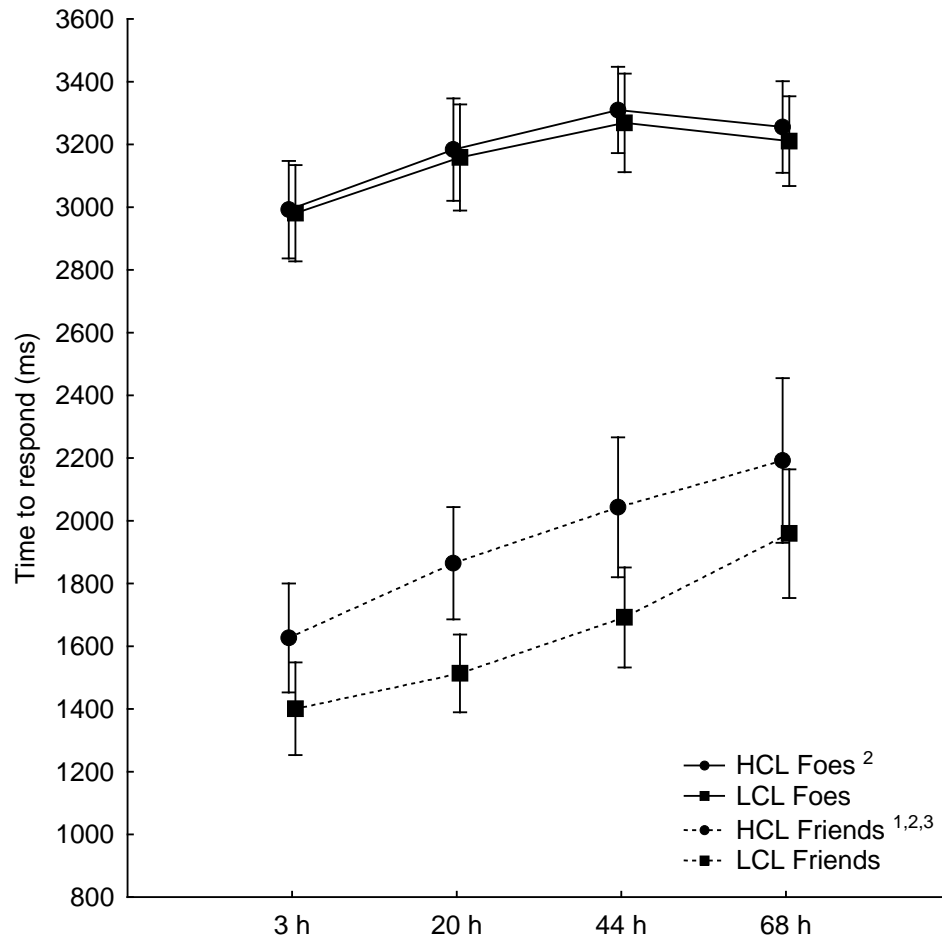


Figure 11. Time to shoot a foe target and time to signal a friendly target in each of the cognitive load conditions.

The x-axis is the number of elapsed hours from the start of the experiment. Bars denote standard error. ¹Task Condition $P < 0.05$, ²Time $P < 0.05$, ³Challenge Order, $P < .05$. Refer to the results section for detailed descriptions of significant differences.

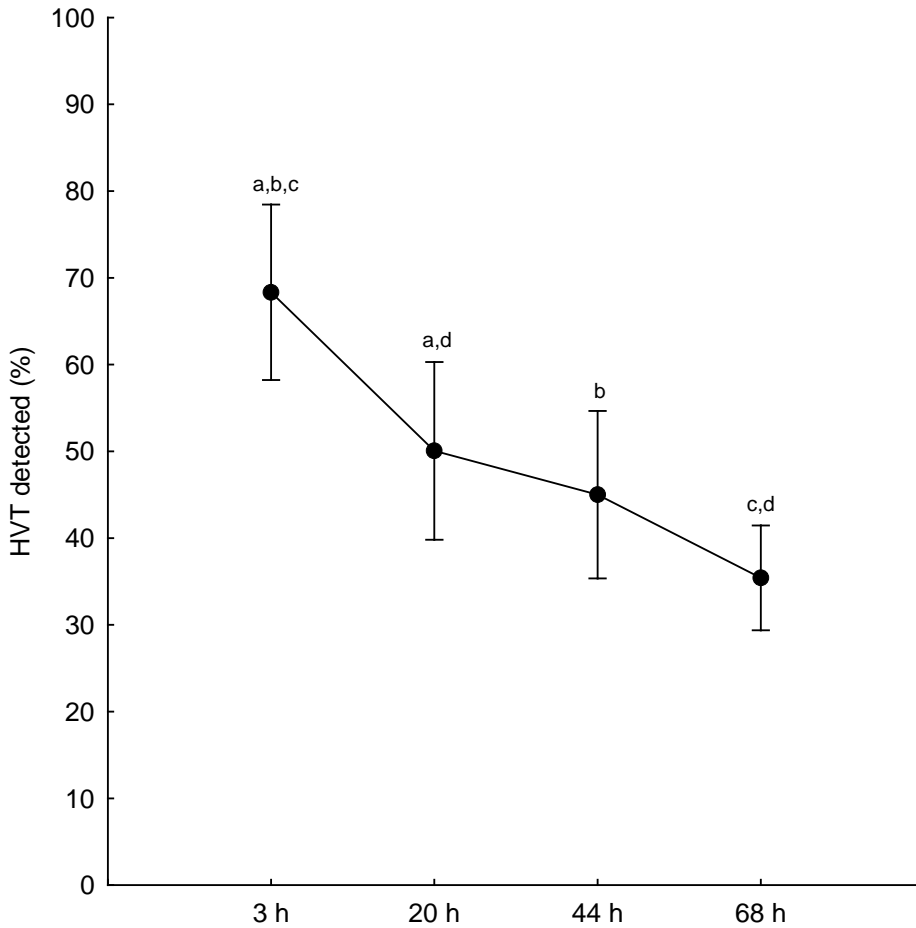


Figure 12. Percentage of foe targets accurately hit and percentage of correct responses (shots at foes plus button presses on friends; excluding omitted responses) in each of the cognitive load conditions.

The x-axis is the number of elapsed hours from the start of the experiment. Bars denote standard error.

¹Task Condition x Time $P < .05$, ²Task Condition $P < .05$. Time points that share common letters are significantly different from each other. Refer to the results section for detailed descriptions of significant differences.

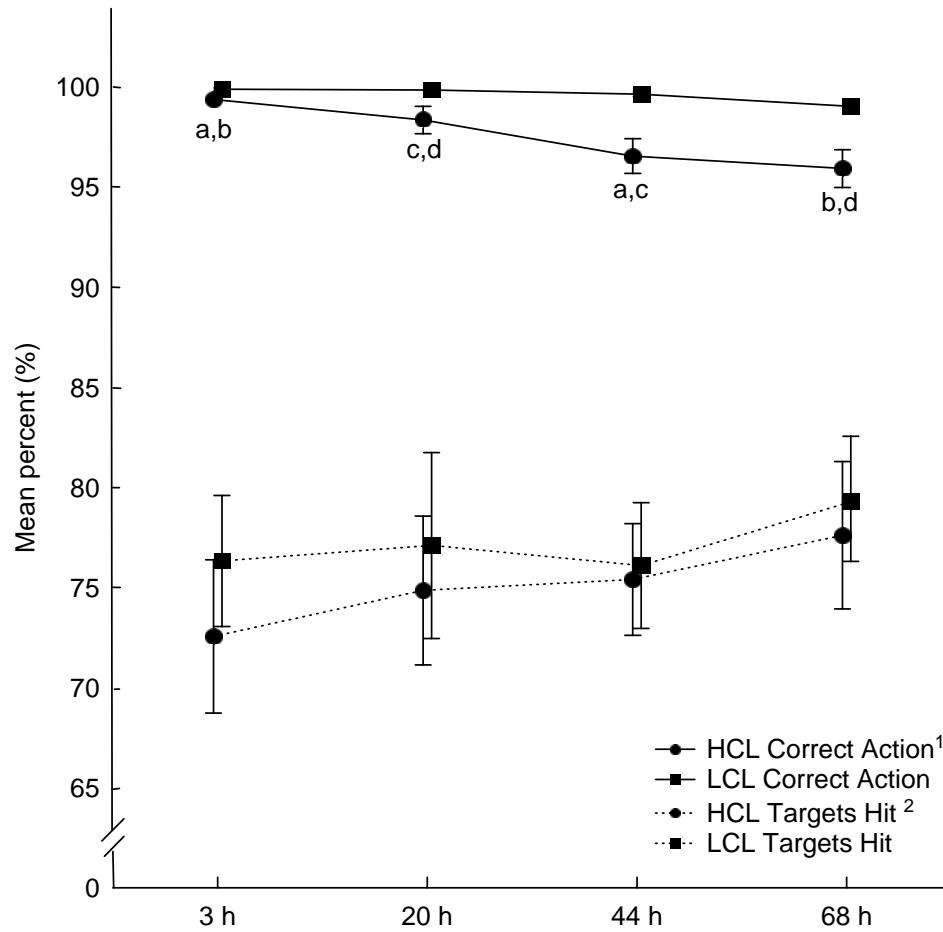


Figure 13. The percentage of correctly detected high value targets (HVT) during the high cognitive load condition.

The x-axis is the number of elapsed hours from the start of the experiment. Bars denote standard error. Time points that share common letters are significantly different from each other at $P < .05$.